

<p>(51) International Patent Classification <sup>6</sup> :  <b>C07F 7/18, A61K 9/127, 31/695</b></p>	<p><b>A1</b></p>	<p>(11) International Publication Number: <b>WO 95/21175</b></p> <p>(43) International Publication Date: <b>10 August 1995 (10.08.95)</b></p>
<p>(21) International Application Number: <b>PCT/US95/01490</b></p> <p>(22) International Filing Date: <b>2 February 1995 (02.02.95)</b></p> <p>(30) Priority Data:  08/190,295                      2 February 1994 (02.02.94)                      US</p> <p>(71) Applicant: <b>THE LIPOSOME COMPANY, INC. [US/US];</b> One Research Way, Princeton Forrestal Center, Princeton, NJ 08540 (US).</p> <p>(72) Inventors: <b>PEI, Yong-Wei;</b> 39 Seneca Terrace, Branchburg, NJ 08876 (US). <b>MAYHEW, Eric;</b> One Research Way, Princeton Forrestal Center, Princeton, NJ 08540 (US). <b>AHMAD, Imran;</b> 2408 Fox Run Drive, Plainsboro, NJ 08536 (US). <b>JANOFF, Andrew, S.;</b> 1807 South Crescent Boulevard, Yardley, PA 19067 (US).</p> <p>(74) Agent: <b>RUBIN, Kenneth, B.;</b> The Liposome Company, Inc., One Research Way, Princeton Forrestal Center, Princeton, NJ 08540 (US).</p>		<p>(81) Designated States: <b>AU, CA, FI, JP, KR, NO,</b> European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p><b>Published</b>  <i>With international search report.</i></p>
<p>(54) Title: <b>PHARMACEUTICALLY ACTIVE COMPOUNDS AND LIPOSOMES, AND METHODS OF USE THEREOF</b></p>		
<p>The diagram illustrates the metabolic pathway of sphingomyelin. At the top, the chemical structure of Sphingomyelin (SM) is shown, consisting of a long-chain fatty acid, a sphingosine backbone, and a phosphocholine head group. An arrow labeled 'reversible hydrolysis' with a question mark and 'sphingomyelinase' indicates its conversion to Ceramide (Cer). The Ceramide structure shows the removal of the phosphate group. From Ceramide, two arrows branch out: one labeled 'deacylation' leading to the structure of Sphingoid base (labeled as inhibitors of protein kinase C), and another leading to 'Protein Kinase'.</p>		
<p>(57) Abstract</p> <p>This invention provides a compound having the formula <math>R^1-Y^1-CHZ^1-CH(NY^2Y^3)-CH_2-Z^2</math>, wherein: <math>R^1</math> is a straight-chained alkyl, alkenyl or alkynyl group having from 8 to 19 carbon atoms in the aliphatic chain; <math>Y^1</math> is <math>-CH=CH-</math>, <math>-C\equiv C-</math> or <math>-CH(OH)CH(OH)-</math>; <math>Z^1</math> is OH or a conversion-inhibiting group; <math>Z^2</math> is a conversion-inhibiting group; <math>Y^2</math> is H, a phenyl group, an alkyl-substituted phenyl group having from 1 to about 6 carbons in the alkyl chain, or an alkyl chain having from 1 to 6 carbons; <math>Y^3</math> is H or a group having the formula <math>-C(O)R^2</math> or <math>-S(O)_2R^2</math>; <math>R^2</math> is a straight-chained alkyl, alkenyl or alkynyl group having from 1 to 23 carbon atoms in the chain; and when <math>Z^2</math> is an amino, <math>R^2</math> is an aliphatic chain having from 1 to 9 or from 19 to 23 carbon atoms in the aliphatic chain.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

**PHARMACEUTICALLY ACTIVE COMPOUNDS AND LIPOSOMES,  
AND METHODS OF USE THEREOF**

5    **Field of the Invention**

          This invention is directed to pharmaceutically active sphingolipid compounds, to liposomes containing pharmaceutically active sphingolipid compounds, and to methods of using such compounds and liposomes, particularly for the treatment of animals afflicted with cancers.

10           Cell death in multicellular organisms can be an accidental response to external trauma, or it can be a programmed response to internal or external stimuli. Necrosis, or accidental cell death, is most often seen when cells die uncontrollably as a result of sudden and severe injury to an organism, e.g., by physical or chemical trauma, sustained hyperthermia or ischemia (see, e.g., J. Cohen, *Immunol. Today*, 14(3):126 (1993)); J. Marx, *Science* 259: 750 (1993)). Plasma  
15    membrane damage can cause cells to lose their ability to regulate their osmotic pressure, and cell rupture can thereby result. The consequent leakage of cell contents can cause further injury to surrounding cells and can invoke an inflammatory response to clear away the cellular debris.

20           Apoptosis, by contrast, describes a programmed series of events resulting in cell death by fragmentation into membrane-bound particles; these particles are then phagocytosed by other cells (see, e.g., Stedman's Medical Dictionary (Illustrated), *supra*). Cells typically undergo apoptosis in physiologically determined circumstances such as the elimination of self-reactive T cells, the death of cells (e.g., neutrophils) with short half-lives, involution of growth factor-  
25    deprived cells, morphogenetic cell death during embryonic development and the deaths of cellular targets of cell-mediated cytotoxicity (see, e.g., J. Cohen, *supra*).

          Cells undergoing apoptosis can break up into apoptotic bodies, which are cellular fragments that retain their membranes and are able to regulate their osmotic pressures. Unlike  
30    necrotic cells, there is usually no leakage of cellular contents and hence, no invocation of an inflammatory response. Apoptotic cells typically have disrupted plasma membranes and condensed, disrupted nuclei. Nuclear chromatin in these cells is fragmented randomly between nucleosomes, as the result of endonuclease activation during apoptosis.

35           Although transcription in apoptotic cells ceases, cell death occurs more rapidly than would be expected from the cessation of transcription alone. This indicates that cellular processes in addition to transcription termination are likely to be involved in apoptosis. Gene expression itself may actually be required for the occurrence of the morphological changes

associated with apoptosis (see, e.g., J. Cohen, *supra*). Alternatively, inhibition of transcription termination may itself induce apoptosis. Furthermore, apoptosis of some cells does not appear to be affected one way or the other by the inhibition of protein synthesis. Expression of the bcl-2 oncogene, for example, can inhibit the apoptosis otherwise induced by different stimuli, and may thereby contribute to cancer development. Accordingly, inhibition of bcl-2 expression may be required to induce apoptosis (see, e.g., J. Marx, *supra*; J. Cohen, *supra*; G. Williams and C. Smith, *Cell* 74:777 (1993); M. Barinaga, *Science* 259: 762 (February 5, 1993)). C-myc protein is known to stimulate cell proliferation; however, it may also stimulate apoptosis in the absence of additional proliferative stimuli. p53, which is thought to suppress tumor growth, may also stimulate apoptosis. C-fas, a transmembrane protein homologous to Tumor Necrosis Factor (TNF), can also induce apoptosis, as can TNF itself.

TNF is a monokine protein produced by monocytes and macrophages. There are two known structurally and functionally related TNF proteins, TNF- $\alpha$  and TNF- $\beta$ , both of which bind to the same cell surface receptors. Binding to these receptors by TNF leads to the activation of multiple signal transduction pathways, including the activation of sphingomyelinase (see, e.g., M. Raines et al., *J. Biol. Chem.* 268(20):14572 (1993); L. Obeid et al., *Science* 259:1769 (March 12, 1993); H. Morishige et al., *Biochim. Biophys. Acta.* 1151:59 (1993); J. Vilcek and T. Lee, *J. Biol. Chem.* 266(12):7313 (1991); Dbaibo et al., *J. Biol. Chem.* 268(24):17762 (1993); R. Kolesnik, *Trends Cell. Biol.* 2:232 (1992); J. Fishbein et al., *J. Biol. Chem.* 268(13):9255 (1993)).

Applicants have found that increases in ceramide concentrations can stimulate apoptosis. Ceramides are a class of sphingolipids comprising fatty acid derivatives of a sphingoid, e.g., sphingosine, base (see, e.g., Stedman's Medical Dictionary (Illustrated), 24th edition (J. V. Basmajian et al., eds.), Williams and Wilkins, Baltimore (1982), p. 99)). Different ceramides are characterized by different fatty acids linked to the sphingoid base. For example, stearic acid can be attached to the amide group of sphingosine to give rise to the ceramide  $\text{CH}_3(\text{CH}_2)_{12}\text{CH}=\text{CH}-\text{CHOH}-\text{CH}(\text{CH}_2\text{OH})-\text{NH}-\text{CO}-(\text{CH}_2)_{16}\text{CH}_3$ . Shorter- or longer-chain fatty acids can also be linked to the sphingoid base. Applicants have also found that attachment of certain chemical groups to sphingolipids and ceramides so as to form analogs of such compounds can inhibit bioconversion of ceramides to sphingomyelins, and can thereby lead to an apoptosis stimulating increase in ceramide concentrations.

Ceramides are found in all eukaryotic cell membranes, and are known to participate in a variety of critical cellular processes. Furthermore, certain sphingolipid compounds have been found to play a role in prevention of cellular proliferation (). However, none of these references teach applicants' chemical compounds and liposomes, or their use in stimulating cell death.

**SUMMARY OF THE INVENTION**

5        Provided herein is a compound having the formula  $R^1-Y^1-CHZ^1-CH(NY^2Y^3)-CH_2-Z^2$ , wherein:  $R^1$  is a straight-chained alkyl, alkenyl or alkynyl group having from 8 to 19 carbon atoms in the aliphatic chain;  $Y^1$  is  $-CH=CH-$ ,  $-C\equiv C-$  or  $-CH(OH)CH(OH)-$ ;  $Z^1$  is OH or a conversion-inhibiting group;  $Z^2$  is a conversion-inhibiting group;  $Y^2$  is H, a phenyl group, an alkyl-substituted phenyl group having from 1 to about 6 carbons in the alkyl chain, or an alkyl chain having from 1 to 6 carbons;  $Y^3$  is H or a group having the formula  $-C(O)R^2$  or  $-S(O)_2R^2$ ;  $R^2$  is a straight-chained alkyl, alkenyl or alkynyl group having from 1 to 23 carbon atoms in the chain; and wherein when  $Z^2$  is an amino,  $R^2$  is an aliphatic chain having from 1 to 9 or from 19 to 23 carbon atoms in the aliphatic chain. Preferably,  $R^1$  is an alkyl group, more preferably,  $CH_3(CH_2)_{12}-$ ,  $Y^1$  is  $-CH=CH-$ ,  $Y^2$  is H,  $Y^3$  is  $-C(O)R^2$  and  $R^2$  is an alkyl chain.

15        Conversion-inhibiting groups can have the formula  $-X^2X^3$  or  $-O-X^2X^3$ , wherein  $X^2$  is selected from the group consisting of  $CH_2-$ ,  $C(CH_3)_2-$ ,  $Si(PO_4)_2-$ ,  $Si(CH_3)_2-$ ,  $SiCH_3PO_4-$ ,  $C(O)-$  and  $S(O)_2-$  and wherein  $X^3$  is selected from the group consisting of  $-C(O)H$ ,  $-CO_2H$ ,  $-CH_3$ ,  $-C(CH_3)_3$ ,  $-Si(CH_3)_3$ ,  $-SiCH_3(C(CH_3)_3)_2$ ,  $-Si(C(CH_3)_3)_3$ ,  $-Si(PO_4)_2C(CH_3)_3$ , a phenyl group, an alkyl-substituted phenyl group having from 1 to 6 carbons in the alkyl chain, an alkyl chain having from 1 to 6 carbons, an amino moiety, a chlorine, a fluorine, or a group having the formula  $C(R^3R^4)OH$ ; each of  $R^3$  and  $R^4$  is independently an alkyl chain having from 1 to 6 carbons, a phenyl group or an alkyl-substituted phenyl group having from 1 to 6 carbons in the alkyl chain. Preferably, the conversion-inhibiting group is  $-OC(O)CH_3$ ,  $-OC(O)CH_2CH_2CH_3$ ,  $-OC(O)CH(CH_3)CH_3$ , or  $-OSi(CH_3)_2C(CH_3)_3$ , more preferably,  $-OSi(CH_3)_2C(CH_3)_3$ . Conversion-inhibiting groups can also have the formula  $-X^1$  or  $-OX^1$ , wherein  $X^1$  is  $C(O)H$ ,  $CO_2H$ ,  $CH_3$ ,  $C(CH_3)_3$ ,  $Si(CH_3)_3$ ,  $SiCH_3(C(CH_3)_3)_2$ ,  $Si(C(CH_3)_3)_3$ ,  $Si(PO_4)_2C(CH_3)_3$ , a phenyl group, an alkyl-substituted phenyl group having from 1 to 6 carbons in the alkyl chain, an alkyl chain having from 1 to 6 carbons, an amino moiety, a fluorine, a chlorine, or a group having the formula  $C(R^3R^4)OH$ , and each of  $R^3$  and  $R^4$  is independently an alkyl chain having from 1 to 6 carbons.

35        Preferably, the compound of this invention has the formula  $CH_3(CH_2)_{12}-CH=CH-CH_2Z^1-CH(NHY^3)-CH_2-Z^2$ .  $Y^3$  is then a group having the formula  $-C(O)R^2$ , more preferably,  $-C(O)(CH_2)_4CH_3$ .  $Z^2$  is preferably  $-OSi(CH_3)_2C(CH_3)_3$ ,  $-OSi(PO_4)_2C(CH_3)_3$ ,  $-C(O)CH_3$  or  $-OC(O)CH_2CH_2CH_3$ .

Also provided herein is a pharmaceutical composition comprising the compound of this invention and a pharmaceutically acceptable carrier; the composition can also comprise an additional bioactive agent. Further provided is a method of administering a bioactive compound to an animal, preferably a human, which comprises administering to the animal this composition; the method can comprise administering an additional bioactive agent to the animal.

The animal can be afflicted with a cancer, wherein the method comprises administering an amount of the composition which comprises an anticancer effective amount of the compound. Typically, the anticancer effective amount of the compound is at least about 0.1 mg of the compound per kg of body weight of the animal. Generally, the anticancer effective amount is from about 1 mg per kg to about 50 mg per kg. Treatable cancers include, without limitation, brain, breast, lung, ovarian, colon, stomach or prostate cancers, and can be sarcomas, carcinomas, neuroblastomas, or gliomas. Drug resistant cancers can also be treated.

Provided herein is a liposome having a bilayer which comprises a lipid and a compound having the formula  $R^1-Y^1-CHZ^1-CH(NY^2Y^3)-CH_2-Z^2$ , wherein:  $R^1$  is a straight-chained alkyl, alkenyl or alkynyl group having from 5 to 19 carbon atoms in the chain;  $Y^1$  is  $-CH=CH-$ ,  $-C\equiv C-$  or  $-CH(OH)CH(OH)-$ ; each of  $Z^1$  and  $Z^2$  is independently OH or a conversion-inhibiting group;  $Y^2$  is H, a phenyl group, an alkyl-substituted phenyl group having from 1 to 6 carbons in the alkyl chain, or an alkyl chain having from 1 to 6 carbons;  $Y^3$  is H or a group having the formula  $-R^2$ ,  $-C(O)R^2$  or  $-S(O)_2R^2$ ;  $R^2$  is a straight-chained alkyl, alkenyl or alkynyl group having from 1 to 23 carbon atoms; and wherein the bilayer comprises at least about five mole percent of the compound.  $Y^3$  is preferably  $R^2$ , which is preferably,  $-(CH_2)_3CH_3$ ,  $-(CH_2)_5CH_3$ ,  $-(CH_2)_7CH_3$ , or  $-(CH_2)_9CH_3$ , and more preferably,  $R^2$  is  $-(CH_2)_5CH_3$ , or  $-C(O)R^2$ , which is preferably  $-C(O)(CH_2)_4CH_3$ . Preferably in the liposome of this invention, at least one of  $Z^1$  and  $Z^2$  is a conversion-inhibiting group, such as  $-OC(O)CH_3$ ,  $-OC(O)CH_2CH_2CH_3$ ,  $-OC(O)CH(CH_3)CH_3$ , or  $-OSi(CH_3)_2C(CH_3)_3$ . More preferably, the conversion-inhibiting group is  $-OSi(CH_3)_2C(CH_3)_3$ . Most preferably, the liposome comprises a compound having the formula  $CH_3-(CH_2)_{12}-CH=CH-CH_2Z^1-CH(NHY^3)-CH_2Z^2$ .

Preferably, the liposomal bilayer comprises at least about 10 mole percent of the compound. The bilayer can comprise vitamin D<sub>3</sub>; such bilayers preferably comprise about 1 mole percent of vitamin D<sub>3</sub>. The bilayer can also comprise a headgroup-modified lipid. The liposome can comprise an additional bioactive agent, and can be dehydrated.

Also provided herein is a pharmaceutical composition comprising the liposome of this invention and a pharmaceutically acceptable carrier. Further provided is a method of administering a compound to an animal which comprises administering to the animal the composition. The method can be used to treat an animal afflicted with a cancer, wherein a  
 5 dose of the composition is administered and wherein the dose comprises an anticancer effective amount of the liposome. Typically, the dose comprises at least about 1 mg of the liposome per kg of body weight of the animal. Generally, the dose comprises from about 1 mg per kg to about 1000 mg per kg.

10 Provided herein is a liposome having a bilayer which comprises a lipid and a compound having the formula  $R^1-Y^1-CHZ^1-CH(NY^2Y^3)-CH_2-Z^2$ , wherein:  $R^1$  is a straight-chained alkyl, alkenyl or alkynyl group having from 5 to 19 carbon atoms in the chain;  $Y^1$  is  $-CH=CH-$ ,  $-C\equiv C-$  or  $-CH(OH)CH(OH)-$ ; each of  $Z^1$  and  $Z^2$  is independently OH or a conversion-inhibiting group;  $Y^2$  is H, a phenyl group, an alkyl-substituted phenyl group having from 1 to 6 carbons or an  
 15 alkyl chain having from 1 to 6 carbons;  $Y^3$  is H or a group having the formula  $-R^2$ ,  $-C(O)R^2$  or  $-S(O)_2R^2$ ;  $R^2$  is a straight-chained alkyl, alkenyl or alkynyl group having from 1 to 23 carbon atoms; and wherein the bilayer comprises an anticancer-effective amount of the compound.  
 Also provided is a pharmaceutical composition comprising this liposome and a pharmaceutically acceptable carrier. Further provided is a method of treating an animal afflicted with a cancer  
 20 which comprises administering to the animal this composition.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

25 **FIGURE 1.** Ceramide Metabolism. Cer: ceramides; SM:

**FIGURE 2.** Ceramides Comprising Conversion-inhibiting Group. A: Type III Cer-1-TBDMS; C2 Cer-1-TBDMS; C6 Cer-1-TBDMS; C2 Cer-1-TBDPS. B: 3-TBDMS C6-Cer; 1-TBDMS C6-Cer; 1,3 DiTBDMS C6 Cer (YWI51.a); 1-TBDMS, 3-acetate C6-Cer; 1-TBDMS,3-butyrate, C6-Cer. C: 1-acetate-3-one C6-Cer; 4,5-diol C6-Cer. D: N-C4 sphingosine; n-hexyl sphingosine; n-C8 sphingosine; N-C10 sphingosine.

**FIGURE 3.** Effect of Various Liposomal Ceramide/ Sphingomyelin Formulations on the Growth of HL-60 Cells. The number of viable cells (per ml x 10,000, y-axis) was determined for  
 35 lipid doses of 100  $\mu$ M and 200  $\mu$ M (z-axis). X-axis: control, egg phosphatidylcholine/cholesterol (EPC/Chol), EPC/Chol/C2-ceramide (C2), EPC/Chol/vitamin D3 (D3), EPC/Chol/D3/C2, EPC/Chol/C6-ceramide (C6), EPC/Chol/D3/C6, SM/Chol and SM/Chol/D3 liposomes.

**FIGURE 4.** Effect of Various Liposomal Ceramide/ Sphingomyelin Formulations on the Growth of P388 Cells. The number of viable cells (per ml x 10,000, y-axis) was determined for lipid doses of 50  $\mu$ M, 100  $\mu$ M and 200 $\mu$ M (z-axis). X-axis: control, egg phosphatidylcholine/ cholesterol (EPC/Chol), EPC/Chol/C2-ceramide (C2), EPC/Chol/vitamin D3 (D3),  
5 EPC/Chol/D3/C2, EPC/Chol/C6-ceramide (C6), EPC/Chol/D3/C6, Sphingomyelin (SM)/Chol and SM/Chol/D3 liposomes.

**FIGURE 5.** Effect of Various Liposomal Ceramide/ Sphingomyelin Formulations on the Growth of U937 Cells. The number of viable cells (per ml x 10,000, y-axis) was determined for  
10 lipid doses of 50  $\mu$ M, 100  $\mu$ M and 200 $\mu$ M (z-axis). X-axis: control, egg phosphatidylcholine/ cholesterol (EPC/Chol), EPC/Chol/C2-ceramide (C2), EPC/Chol/vitamin D3 (D3), EPC/Chol/D3/C2, EPC/Chol/C6-ceramide (C6), EPC/Chol/D3/C6, Sphingomyelin (SM)/Chol and SM/Chol/D3 liposomes.

**FIGURE 6.** Effect of Various Liposomal Ceramide/ Sphingomyelin Formulations on the Growth of RPMI-7666 Cells. The number of viable cells (per ml x 10,000, y-axis) was determined for a lipid dose of 200  $\mu$ M. X-axis: control, egg phosphatidylcholine/ cholesterol (EPC/Chol), EPC/Chol/C2-ceramide (C2), EPC/Chol/vitamin D3 (D3), EPC/Chol/D3/C2,  
15 EPC/Chol/C6-ceramide (C6), EPC/Chol/D3/C6, Sphingomyelin (SM)/Chol and SM/Chol/D3 liposomes.  
20

**FIGURE 7.** Effect of Liposomal Ceramide on the Growth of Normal (RPMI-7666) and Cancer (U-937) Cells. The number of viable cells in each of the cultures was determined and is given as the percent relative to the control (y-axis).  
25

**FIGURE 8.** Therapeutic Efficacy of Liposomal Ceramides in Mice. X-axis: days post-liposome/control administration; y-axis: percent survival in treatment group. Circle-in-square: control mice administered HEPES buffered saline; filled square: liposomal vitamin D3; open diamond: liposomal C2 ceramide; filled diamond: liposomal C6 ceramide.  
30

**FIGURE 9.** *In Vitro* Sensitivity of A549 Cells to C6-Ceramide Derivatives. A: one-hour exposure; B: four-hour exposure; C: eight-hour exposure; D: twenty four-hour exposure; E: forty eight-hour exposure X-axis: drug (ceramide derivative) concentration (micromolar); y-axis: percent cell growth. "X": N-C8 sphingosine; diamond: 1-acetate, C6-ceramide; square: 1-butyrate, C6-ceramide; triangle: 1-isobutyrate, C6-ceramide.  
35

**FIGURE 10.** *In Vitro* Sensitivity of A549 Cells to 1-Acetate, C6-Ceramide for Different Lengths of Exposure. X-axis: drug concentration (micromolar); y-axis: percent growth.



Diamond: one hour; square: four hours; triangle: eight hours; X: twenty four hours; star: forty eight hours.

**FIGURE 11.** *In Vitro* Sensitivity of A549 Cells to 1-Butyrate, C6-Ceramide for Different Lengths of Exposure. X-axis: drug concentration (micromolar); y-axis: percent growth. Diamond: one hour; square: four hours; triangle: eight hours; X: twenty four hours; star: forty eight hours.

**FIGURE 12.** *In Vitro* Sensitivity of A549 Cells to 1-Isobutyrate, C6-Ceramide for Different Lengths of Exposure. X-axis: drug concentration (micromolar); y-axis: percent growth. Diamond: one hour; square: four hours; triangle: eight hours; X: twenty four hours; star: forty eight hours.

**FIGURE 13.** *In Vitro* Sensitivity of A549 Cells to N-C8 Sphingosine for Different Lengths of Exposure. X-axis: drug concentration (micromolar); y-axis: percent growth. Diamond: one hour; square: four hours; triangle: eight hours; X: twenty four hours; star: forty eight hours.

**FIGURE 14.** Therapeutic Efficacy of N-hexyl Sphingosine Against P-388/ADR (adriamycin-resistant) Tumor-Bearing Mice. X-axis: days after therapy; y-axis: percent survival in treatment group. "+": Control (untreated); squares: one dose of 20 mg n-hexyl sphingosine per kg of body weight; circles: 3 20 mg/kg doses.

## 25 **DETAILED DESCRIPTION OF THE INVENTION**

Provided herein is a compound having the formula  $R^1-Y^1-CHZ^1-CH(NY^2Y^3)-CH_2-Z^2$ , wherein:  $R^1$  is a straight-chained alkyl, alkenyl or alkynyl group having from 8 to 19 carbon atoms in the aliphatic chain;  $Y^1$  is  $-CH=CH-$ ,  $-C\equiv C-$  or  $-CH(OH)CH(OH)-$ ;  $Z^1$  is OH or a conversion-inhibiting group;  $Z^2$  is a conversion-inhibiting group;  $Y^2$  is H, a phenyl group, an alkyl-substituted phenyl group having from 1 to about 6 carbons in the alkyl chain, or an alkyl chain having from 1 to 6 carbons;  $Y^3$  is H or a group having the formula  $-C(O)R^2$  or  $-S(O)_2R^2$ ;  $R^2$  is a straight-chained alkyl, alkenyl or alkynyl group having from 1 to 23 carbon atoms in the chain; and wherein when  $Z^2$  is an amino,  $R^2$  is an aliphatic chain having from 1 to 9 or from 19 to 23 carbon atoms in the aliphatic chain. Preferably,  $R^1$  is an alkyl group, more preferably,  $CH_3(CH_2)_{12}-$ ,  $Y^1$  is  $-CH=CH-$ ,  $Y^2$  is H,  $Y^3$  is  $-C(O)R^2$  and  $R^2$  is an alkyl chain, more preferably an alkyl chain having from 6 to 8 carbons. Most preferably,  $R^1$  and  $R^2$  together comprise from about 15 to about 25 carbons, wherein  $R^1$  preferably comprises 13 carbons and  $R^2$  preferably comprises 6 to 8 carbons. Without intending to be limited by theory, it is believed that total

carbon chain length of a lipid is an important factor in determining the ability of the lipid to insert itself into biological membranes.

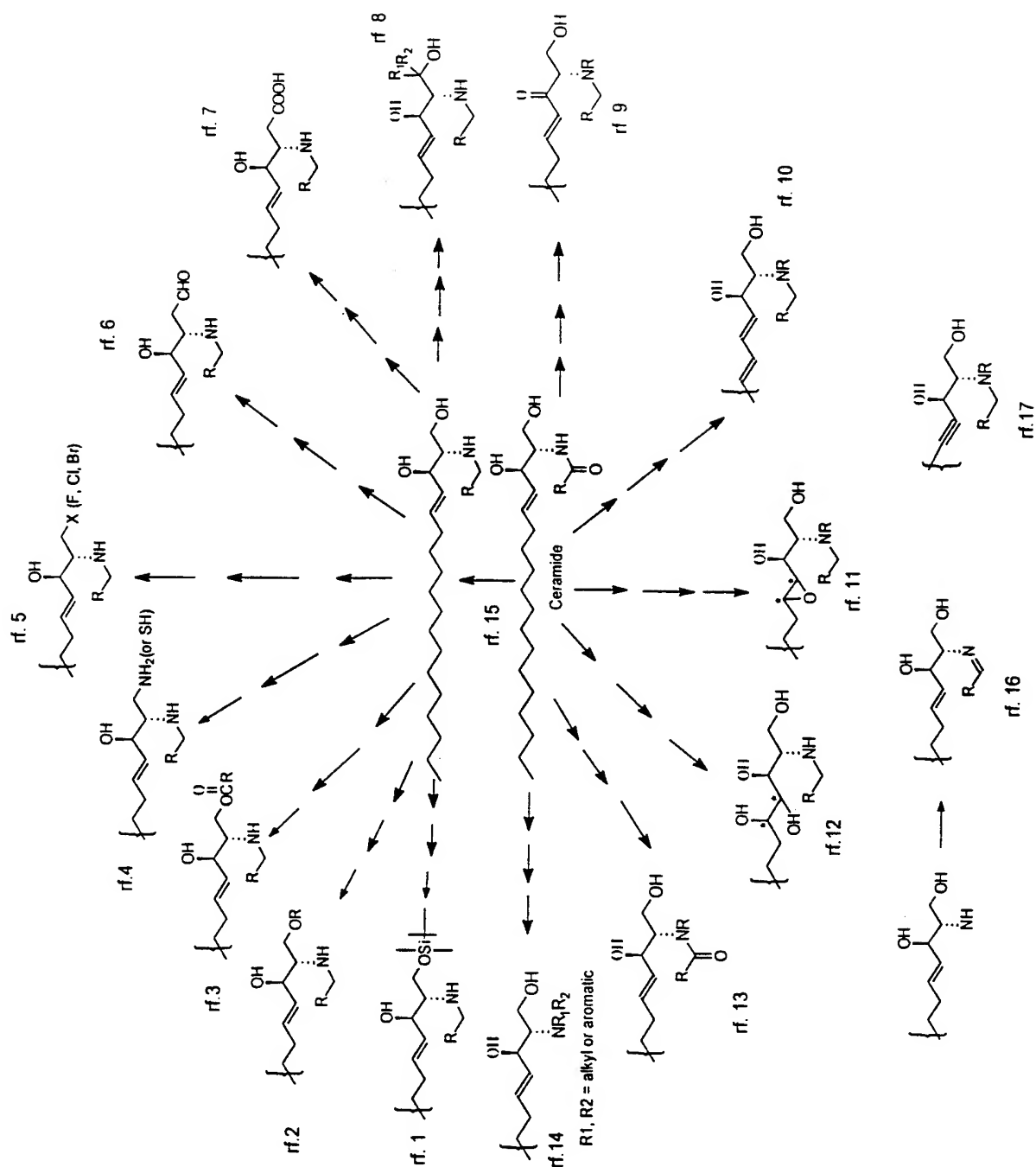
Without intending for this invention in any way to be limited by theory, it is believed that sphingosines and ceramides can act as signal transducers or secondary messengers in cells, i.e., that intracellular levels are increased in response to external stimuli and that this increase results in enhanced protein kinase and phosphatase activities (see, e.g., M. Raines et al., *supra*; R. Kolesnik et al., *supra*; G. Dbaibo et al., *supra*; and J. Fishbein et al., *supra*). Activated protein kinases and phosphatases can activate cellular processes which lead to cell death. Accordingly, it can be therapeutically desirable to increase intracellular concentrations of sphingosines and ceramides in cancer cells.

Sphingosines and ceramides are formed in animal cells by the combination of palmitoyl CoA ( $\text{CH}_3(\text{CH}_2)_{14}\text{-CO-S-CoA}$ ) and serine to give dehydrosphinganine ( $\text{CH}_3(\text{CH}_2)_{14}\text{CH}(\text{NH}_3)\text{-CH}_2\text{OH}$  and  $\text{CO}_2$  (see, e.g., L. Stryer, *Biochemistry* (2nd edition), W. H. Freeman and Co., New York, pp. 461-462)). Dehydrosphinganine is converted to dihydrosphingosine ( $\text{CH}_3(\text{CH}_2)_{14}\text{-CH(OH)-CH(NH}_3\text{)-CH}_2\text{OH}$ ) which is then converted to sphingosine ( $\text{CH}_3(\text{CH}_2)_{12}\text{CH=CH-CH(OH)-CH(NH}_3\text{)-CH}_2\text{OH}$ ). A fatty acid is then linked to the amide group of sphingosine to give rise to a ceramide ( $\text{CH}_3(\text{CH}_2)_{12}\text{CH=CH-CHOH-CH(CH}_2\text{OH)-NH-CO-R}$ , where R is a fatty acid chain). A phosphorylcholine group ( $\text{PO}_4\text{CH}_2\text{CH}_2\text{-N(CH}_3\text{)}_3$ ) can be attached to the ceramide at its hydroxyl group to produce a sphingomyelin ( $\text{CH}_3(\text{CH}_2)_{12}\text{CH=CH-CHOH-CH(CH}_2\text{PO}_4\text{CH}_2\text{CH}_2\text{-N(CH}_3\text{)}_3\text{)-NH-CO-R}$ ). Sphingomyelinase can catalyze the hydrolytic removal of the phosphorylcholine from the sphingomyelin to give rise to a ceramide (see, e.g., Figure 1). Reverse hydrolysis of the ceramide can give rise to a sphingomyelin.

Blockage or inhibition of this "reverse hydrolysis" step, that is, conversion of a ceramide to the corresponding sphingomyelin, can lead to increased intracellular ceramide levels. "Conversion-inhibiting groups" are generally not groups found attached to sphingosines or ceramides in animal cells, or to their biosynthetic precursors. Rather, such groups are synthetically attached to sphingosines and ceramides to inhibit sphingomyelin formation therefrom.

The compounds of this invention, comprising conversion-inhibiting groups and alkyl, alkenyl, alkynyl chains of varying length, are synthesized by a number of routes well known to and readily practiced by ordinarily skilled artisans, given the teachings of this Invention (see, for example, below, wherein "rf" refers to one of the following references: 1: J. Am. Chem. Soc., 94: 6190 (1972); 2: J. Org. Chem. 59: 668 (1994); 3: Angew. Chem., Intl. Ed. (English), 17: 569

- (1978); 4: Vogel's Textbook of Practical Organic Chemistry (5th ed.), pp. 769-780); 5: J. Org. Chem. 40: 574 (1975); 6: J. Org. Chem. 59: 182 (1994); 7: J. Org. Chem. 25: 2098 (1960); 8: Synthesis (1985): pp. 253-268; 9: J. Chem. Soc. (1953): p. 2548; 10: J. Am. Chem. Soc. 90: 4462, 4464 (1968); 11: Oxidations in Organic Chemistry (Am. Chem. Soc, Washington, D.C. (1990), pp. 60-64; 12: J. Med. Chem. 30: 1326 (1987); 13: Synth. Commun. 9: 757 (1979); 14: The Chemistry of Amides (J. Wiley & Sons, New York (1970)), pp. 795-801; 15: J. Med. Chem. 37: 2896 (1994); 16: J. Med. Chem. 30: 1326 (1987); 16: Rec. Chem. Prog. 29: 85 (1968); and 17: Phospholipids Handbook (Marcell Dekker, Inc., New York (1993), p. 97)). For example, such artisans would use a sphingosine or a ceramide as their starting material. Alkyl, alkenyl or alkynyl chains of varying length can be attached thereto, or removed therefrom, by known means. Conversion-inhibiting groups can also be attached to the sphingosines and ceramides by known means. These include, without limitation, oxidation/reduction, substitution, condensation and alkylation reactions, as well as other generally accepted means for attaching and removing chemical groups from a compounds and for converting compounds from one form to another. Such reactions are generally formed using generally accepted solvents and are performed under readily determinable conditions.



Specific compounds can be synthesized as follows. Synthesis of sily ether of ceramide:

The mixture of Ceramide and t-Butyldimethylsilyl chloride ( 1 equivalent) and imidazole ( 2 equivalent) in DMF was stirred under N<sub>2</sub> at room temperature overnight. The solvent was removed under a stream of N<sub>2</sub> and residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub>, washed (H<sub>2</sub>O), dried (MgSO<sub>4</sub>) and concentrated to dryness. The residue was purified over silica gel (AcOEt: Hexan=1:3). Synthesis of 1-ester ceramide: The mixture of ceramide and Ac<sub>2</sub>O ( 1 equivalent) and catalytic amount of dimethyl amino pyridine in dry CH<sub>2</sub>Cl<sub>2</sub> was stirred at room temperature for 1 hour and the reaction was checked by TLC (AcOEt). The mixture was concentrated. The crude product was purified over silica gel (AcOEt: Hexane = 2:3.5) Oxidation of C3-OH of ceramide to ketone: 1-OAc ceramide was dissolved in acetone and cooled in ice-bath. Jones' reagent was dropwised slowly till the orange color persistent. The reaction was quenched by isopropanol, and NaHCO<sub>3</sub> was added and stirred for 5 minutes. The solution was filtrate and concentrated to dryness. The crude product was purified by preparative TLC (ACOET: Hexane = 1:2.5). Reduction of ceramide to sphingosine analogs: To an ice-cold stirred solution of ceramide in anhydrous THF was added LiAlH<sub>4</sub> and the mixture was stirred at room temperature under N<sub>2</sub> for 24 hours. Under ice cooling the reaction mixtures was quenched by addition of saturated aqueous NaHCO<sub>3</sub>. The resulting slurry was filtered and washed with THF. The solution was concentrated and the residue was brought into CH<sub>2</sub>Cl<sub>2</sub>, washed with H<sub>2</sub>O, dried (MgSO<sub>4</sub>) and concentrated to dryness. The residue was purified over preparative TLC (silica gel) CH<sub>2</sub>Cl<sub>2</sub> : MeOH : TEA=8 :1 : 0.08. Synthesis of 4,5-diol ceramide: To a solution of ceramide in a mixture of Me<sub>2</sub>CO distilled H<sub>2</sub>O and t-BuOH, N-Methyl morpholine N-oxide (NMO, 1.2 equivalent) and OsO<sub>4</sub> (catalytic amount) in THF were added. The reaction mixture was stirred at 45°C for 6 hours and it was quenched by solid NaHCO<sub>3</sub> and the mixture was stirred for 15 minutes. The suspension was filtered and filtrate was dissolved in THF. The solution was washed with brine. The organic solution was separated , dried and concentrated to dryness. The residue was purified over preparative TLC (THF).

Suitable conversion-inhibiting groups can be identified by a number of means readily practiced by ordinarily skilled artisans, given the motivation by this invention to identify such groups. For example, and without limitation, such artisans can select a chemical moiety, and attach it to a sphingosine or ceramide as described above. The artisans can then readily determine the relative rate at which such a compound undergoes hydrolysis, and the rate at which a sphingomyelin is formed from the compound. Rates of hydrolysis are themselves readily determinable by ordinarily skilled artisans, for example and without limitation, by attaching a radioactive moiety to a sphingosine or ceramide and then following the rate of hydrolytic cleavage of the moiety by chromatographic means. Rates of sphingomyelin formation are also readily determinable, for example and without limitation, by combining radioactive phosphorylcholine with a conversion-inhibiting group-containing compound in an enzyme

system capable of attaching the phosphorylcholine to the compound, and then using chromatographic means to assess the rate at which the phosphorylcholine is added. Preferred conversion-inhibiting groups are those which most inhibit hydrolysis and phosphorylcholine attachment.

5

Conversion-inhibiting groups can have the formula  $-X^2X^3$  or  $-O-X^2X^3$ , wherein  $X^2$  is selected from the group consisting of  $CH_2-$ ,  $C(CH_3)_2-$ ,  $Si(PO_4)_2-$ ,  $Si(CH_3)_2-$ ,  $SiCH_3PO_4-$ ,  $C(O)-$  and  $S(O)_2-$  and wherein  $X^3$  is selected from the group consisting of  $-C(O)H$ ,  $-CO_2H$ ,  $-CH_3$ ,  $-C(CH_3)_3$ ,  $-Si(CH_3)_3$ ,  $-SiCH_3(C(CH_3)_3)_2$ ,  $-Si(C(CH_3)_3)_3$ ,  $-Si(PO_4)_2C(CH_3)_3$ , a phenyl group, an alkyl-substituted phenyl group having from 1 to 6 carbons in the alkyl chain, an alkyl chain having from 1 to 6 carbons, an amino moiety, a chlorine, a fluorine, or a group having the formula  $C(R^3R^4)OH$ ; each of  $R^3$  and  $R^4$  is independently an alkyl chain having from 1 to 6 carbons, a phenyl group or an alkyl-substituted phenyl group having from 1 to 6 carbons in the alkyl chain. Preferably, the conversion-inhibiting group is  $-OC(O)CH_3$ ,  $-OC(O)CH_2CH_2CH_3$ ,  $-OC(O)CH(CH_3)CH_3$ , or  $-OSi(CH_3)_2C(CH_3)_3$  (TBDMS) more preferably,  $-OSi(CH_3)_2C(CH_3)_3$ . Conversion-inhibiting groups can also have the formula  $-X^1$  or  $-OX^1$ , wherein  $X^1$  is  $C(O)H$ ,  $CO_2H$ ,  $CH_3$ ,  $C(CH_3)_3$ ,  $Si(CH_3)_3$ ,  $SiCH_3(C(CH_3)_3)_2$ ,  $Si(C(CH_3)_3)_3$ ,  $Si(PO_4)_2C(CH_3)_3$ , a phenyl group, an alkyl-substituted phenyl group having from 1 to 6 carbons in the alkyl chain, an alkyl chain having from 1 to 6 carbons, an amino moiety, a fluorine, a chlorine, or a group having the formula  $C(R^3R^4)OH$ , and each of  $R^3$  and  $R^4$  is independently an alkyl chain having from 1 to 6 carbons. Accordingly, conversion-inhibiting groups include attachment of chemical moieties to sphingosines and ceramides by ether, silyl ether, ester, acetal and sulfonate linkages.

Preferably, the compound of this invention has the formula  $CH_3(CH_2)_{12}-CH=CH-CH_2Z^1-CH(NHY^3)-CH_2-Z^2$ .  $Y^3$  is then preferably a group having the formula  $-C(O)R^2$ , more preferably,  $-C(O)(CH_2)_4CH_3$ .  $Z^2$  is preferably  $-OSi(CH_3)_2C(CH_3)_3$ ,  $-OSi(PO_4)_2C(CH_3)_3$ ,  $-C(O)CH_3$  or  $-OC(O)CH_2CH_2CH_3$ .

Also provided herein is a pharmaceutical composition comprising the compound of this invention and a pharmaceutically acceptable carrier; the composition can also comprise an additional bioactive agent. "Pharmaceutically acceptable carriers" as used herein are generally intended for use in connection with the administration of lipids and liposomes, including liposomal bioactive agent formulations, to animals, including humans. Pharmaceutically acceptable carriers are generally formulated according to a number of factors well within the purview of the ordinarily skilled artisan to determine and account for, including without limitation: the particular liposomal bioactive agent used, its concentration, stability and intended bioavailability; the disease, disorder or condition being treated with the liposomal composition; the subject, its age, size and general condition; and the composition's intended route of

administration, e.g., nasal, oral, ophthalmic, topical, transdermal, vaginal, subcutaneous, intramammary, intraperitoneal, intravenous, or intramuscular (see, for example, Naim (1985)). Typical pharmaceutically acceptable carriers used in parenteral bioactive agent administration include, for example, D5W, an aqueous solution containing 5% weight by volume of dextrose, and physiological saline. Pharmaceutically acceptable carriers can contain additional ingredients, for example those which enhance the stability of the active ingredients included, such as preservatives and anti-oxidants.

Further provided is a method of administering a bioactive compound to an animal, preferably a human, which comprises administering to the animal this composition; the method can comprise administering an additional bioactive agent to the animal. The administration, which can be by any means generally accepted for administering pharmaceutical products to animals, is generally intravenous administration.

The animal can be afflicted with a cancer, wherein the method comprises administering an amount of the composition which comprises an anticancer effective amount of the compound. Treatable cancers include, without limitation, brain, breast, lung, ovarian, colon, stomach or prostate cancers, and can be sarcomas, carcinomas, neuroblastomas, or gliomas. Drug resistant cancers can also be treated.

"Anticancer effective amounts" of the compounds of this invention are generally amounts effective to inhibit, ameliorate, lessen or prevent establishment, growth, metastasis or invasion of one or more cancers in animals to which the compounds have been administered. Anticancer effective amounts are generally chosen in accordance with a number of factors, e.g., the age, size and general condition of the subject, the cancer being treated and the intended route of administration, and determined by a variety of means, for example, dose ranging trials, well known to, and readily practiced by, ordinarily skilled artisans given the teachings of this invention. Typically, the anticancer effective amount of the compound is at least about 0.1 mg of the compound per kg of body weight of the animal. Generally, the anticancer effective amount is from about 1 mg per kg to about 50 mg per kg.

Provided herein is a liposome having a bilayer which comprises a lipid and a compound having the formula  $R^1-Y^1-CHZ^1-CH(NY^2Y^3)-CH_2-Z^2$ , wherein:  $R^1$  is a straight-chained alkyl, alkenyl or alkynyl group having from 5 to 19 carbon atoms in the chain;  $Y^1$  is  $-CH=CH-$ ,  $-C\equiv C-$  or  $-CH(OH)CH(OH)-$ ; each of  $Z^1$  and  $Z^2$  is independently OH or a conversion-inhibiting group;  $Y^2$  is H, a phenyl group, an alkyl-substituted phenyl group having from 1 to 6 carbons in the alkyl chain, or an alkyl chain having from 1 to 6 carbons;  $Y^3$  is H or a group having the formula  $-R^2$ ,  $-C(O)R^2$  or  $-S(O)_2R^2$ ;  $R^2$  is a straight-chained alkyl, alkenyl or alkynyl group having from 1

to 23 carbon atoms; and wherein the bilayer comprises at least about five mole percent of the compound.

Liposomes are self-assembling structures comprising one or more lipid bilayers, each  
5 of which surrounds an aqueous compartment and comprises two opposing monolayers of  
amphipathic lipid molecules. These comprise a polar (hydrophilic) headgroup region covalently  
linked to one or two non-polar (hydrophobic) acyl chains. Energetically unfavorable contacts  
between the hydrophobic acyl chains and the aqueous medium are generally believed to induce  
lipid molecules to rearrange such that the polar headgroups are oriented towards the aqueous  
10 medium while the acyl chains reorient towards the interior of the bilayer. An energetically stable  
structure is formed in which the acyl chains are effectively shielded from coming into contact  
with the aqueous medium.

Liposomes can be made by a variety of methods (for a review, see, for example,  
15 Deamer and Uster (1983)). These methods include without limitation: Bangham's methods for  
making multilamellar liposomes (MLVs); Lenk's, Fountain's and Cullis' methods for making  
MLVs with substantially equal interlamellar solute distribution (see, for example, U.S. Patent  
Nos. 4,522,803, 4,588,578, 5,030,453, 5,169,637 and 4,975,282); and Paphadjopoulos et al.'s  
reverse-phase evaporation method (U.S. Patent No. 4,235,871) for preparing oligolamellar  
20 liposomes. Unilamellar vesicles can be produced from MLVs by such methods as sonication  
(see Paphadjopoulos et al. (1968)) or extrusion (U.S. Patent No. 5,008,050 and U.S. Patent No.  
5,059,421). The ether lipid liposome of this invention can be produced by the methods of any of  
these disclosures, the contents of which are incorporated herein by reference.

25 Various methodologies, such as sonication, homogenization, French Press application,  
milling and extrusion can be used to size reduce liposomes, that is to prepare liposomes of a  
smaller size from larger liposomes. Tangential flow filtration (see WO89/008846), can also be  
used to regularize the size of liposomes, that is, to produce liposomes having a population of  
liposomes having less size heterogeneity, and a more homogeneous, defined size distribution.  
30 The liposome of this invention can be unilamellar or multilamellar, and preferably has a  
diameter of less than about 200 nm, more preferably, from greater than about 50 nm to less  
than about 200 nm.

Liposomal bilayers can comprise a variety of amphipathic lipids, including those which  
35 are saturated or unsaturated and typically have acyl chains of from 10 to 24 carbons. Suitable  
polar groups include, without limitation, phosphorylcholine, phosphorylethanolamine,  
phosphorylserine, phosphorylglycerol and phosphorylinositol. Suitable acyl chains include,  
without limitation, laurate, myristate, palmitate, stearate and oleate chains. Accordingly,



suitable liposome-forming lipids include, without limitation: egg phosphatidylcholine, distearoyl phosphatidylcholine, dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylethanolamine, dipalmitoyl phosphatidylglycerol and others. Liposomal bilayers can further comprise sterols, such as cholesterol. Sterols generally affect the fluidity of lipid bilayers, typically increasing the fluidity of bilayer hydrocarbon chains below the gel-to-liquid transition temperature ( $T_m$ ), and decreasing fluidity above the  $T_m$  (see, for example, Lewis and McElhaney (1992) and Darnell et al. (1986)). Accordingly, sterol interactions with surrounding hydrocarbon chains generally inhibit emigration of these chains from the bilayer.

Preferably, the liposomal bilayer comprises at least about 10 mole percent of the compound. When  $Y^3$  is  $R^2$ , it is then preferably,  $-(CH_2)_3CH_3$ ,  $-(CH_2)_5CH_3$ ,  $-(CH_2)_7CH_3$ , or  $-(CH_2)_9CH_3$ , and more preferably,  $(CH_2)_5CH_3$ . When  $Y^3$  is  $-C(O)R^2$ , it is then preferably  $-C(O)(CH_2)_4CH_3$ . Preferably, the liposome comprises a compound having the formula  $CH_3-(CH_2)_{12}-CH=CH-CH_2Z^1-CH(NHY^3)-CH_2Z^2$ ; more preferably, the compound comprises at least one conversion-inhibiting group, such as  $-OC(O)CH_3$ ,  $-OC(O)CH_2CH_2CH_3$ ,  $-OC(O)CH(CH_3)CH_3$ , or  $-OSi(CH_3)_2C(CH_3)_3$ . Most preferably, the conversion-inhibiting group is  $-OSi(CH_3)_2C(CH_3)_3$  (TBDMS).

Intracellular ceramide levels can also be increased by administration of vitamin D<sub>3</sub>, either separately from administration of the compounds and liposomes of this invention, or more preferably, in connection with the administration of liposomes. Without intending in any way to be limited by theory, it is believed that vitamin D<sub>3</sub> can stimulate sphingomyelinase to convert sphingomyelins to ceramides. Typically, the bilayer can comprise vitamin D<sub>3</sub>; such bilayers preferably comprise about 1 mole percent of vitamin D<sub>3</sub>. The bilayer can also comprise a headgroup-modified lipid.

The liposome can comprise an additional bioactive agent. A "bioactive agent," is any compound or composition of matter that can be administered to animals, preferably humans. Such agents can have biological activity in animals; the agents can also be used diagnostically in the animals. Bioactive agents include therapeutic and imaging agents. Bioactive agents which may be associated with liposomes include, but are not limited to: antiviral agents such as acyclovir, zidovudine and the interferons; antibacterial agents such as aminoglycosides, cephalosporins and tetracyclines; antifungal agents such as polyene antibiotics, imidazoles and triazoles; antimetabolic agents such as folic acid, and purine and pyrimidine analogs; antineoplastic agents such as the anthracycline antibiotics and plant alkaloids; sterols such as cholesterol; carbohydrates, e.g., sugars and starches; amino acids, peptides, proteins such as cell receptor proteins, immunoglobulins, enzymes, hormones, neurotransmitters and glycoproteins; dyes; radiolabels such as radioisotopes and radioisotope-

labelled compounds; radiopaque compounds; fluorescent compounds; mydriatic compounds; bronchodilators; local anesthetics; and the like. Liposomal bioactive agent formulations can enhance the therapeutic index of the bioactive agent, for example by buffering the agent's toxicity. Liposomes can also reduce the rate at which a bioactive agent is cleared from the circulation of animals. Accordingly, liposomal formulation of bioactive agents can mean that less of the agent need be administered to achieve the desired effect. Additional bioactive agents preferred for the liposome of this invention include antimicrobial, anti-inflammatory and antineoplastic agents, or therapeutic lipids, for example, ceramides. Most preferably, the additional bioactive agent is an antineoplastic agent.

10

Liposomes can be loaded with one or more biologically active agents by solubilizing the agent in the lipid or aqueous phase used to prepare the liposomes. Alternatively, ionizable bioactive agents can be loaded into liposomes by first forming the liposomes, establishing an electrochemical potential, e.g., by way of a pH gradient, across the outermost liposomal bilayer, and then adding the ionizable agent to the aqueous medium external to the liposome (see Bally et al. U.S. Patent No. 5,077,056 and WO86/01102).

15

The liposome of this invention can comprise a headgroup-modified lipid. "Headgroup-modified lipids" are lipids which, when incorporated into the lipid bilayers of liposomes can inhibit clearance of the liposomes from the circulatory systems of animals to which they have been administered. Liposomes are cleared from an animal's body by way of its reticuloendothelial system (RES) which consists of fixed and circulating macrophages. Avoiding RES clearance can allow liposomes to remain in the circulation longer, meaning that less of the drug need be administered to achieve desired serum levels. Enhanced circulation times can also allow targeting of liposomes to non-RES containing tissues. Liposomal surfaces can become coated with serum proteins when administered to animals, i.e., liposomes can be opsonized. Rates of clearance by the RES can be related to the rate and level of opsonization; accordingly, clearance can be inhibited by modifying the outer surface of liposomes such that binding of serum proteins is generally inhibited. This can be accomplished by minimizing or shielding negative surface charges, which can promote protein binding, or by otherwise presenting a steric hindrance to the binding of serum proteins.

25

30

Effective surface modification, that is, alterations to the outer surfaces of liposomes which result in inhibition of opsonization and RES uptake, can be accomplished by incorporating headgroup-modified lipids into liposomal bilayers. "Headgroup-modified lipids" as used herein are amphipathic lipids whose polar headgroups have been derivatized by attachment thereto of a chemical moiety, e.g., polyethylene glycol, a polyalkyl ether, a ganglioside, an organic dicarboxylic acid or the like, which can inhibit the binding of serum proteins to liposomes such

35

that the pharmacokinetic behavior of the vesicles in the circulatory systems of animals is altered (see, e.g., Blume et al., *Biochim. Biophys. Acta.* 1149:180 (1993); Gabizon et al., *Pharm. Res.* 10(5):703 (1993); Park et al. *Biochim. Biophys. Acta.* 1108:257 (1992); Woodle et al., U.S. Patent No. 5,013,556; Allen et al., U.S. Patent Nos. 4,837,028 and 4,920,016; U.S. Serial No. 142,691, filed October 25, 1993; the contents of these disclosures are incorporated herein by reference).

The amount of a headgroup-modified lipid incorporated into the liposome depends upon a number of factors well known to the ordinarily skilled artisan, or within his purview to determine without undue experimentation. These include, but are not limited to: the type of lipid and the type of headgroup modification; the type and size of the liposome; and the intended therapeutic use of the liposomal formulation. Typically, the concentration of the headgroup-modified lipid in the lipid bilayer of the liposome is at least about five mole percent, desirably, about ten mole percent.

The liposome of this invention can be dehydrated, stored and then reconstituted such that a substantial portion of their internal contents are retained in the liposomes. Liposomal dehydration generally requires use of a hydrophilic drying protectant (see U.S. Patent Nos. 4,229,360 and 4,880,635). This hydrophilic compound is generally believed to prevent the rearrangement of the lipids in the liposome, so that the size and contents are maintained during the drying procedure and through rehydration, such that the liposomes can be reconstituted. Appropriate qualities for such drying protectants are that they be strong hydrogen bond acceptors, and possess stereochemical features that preserve the intramolecular spacing of the liposome bilayer components. Saccharide sugars, preferentially mono- and disaccharides, are suitable drying protectants for liposomes. Alternatively, the drying protectant can be omitted if the liposome preparation is not frozen prior to dehydration, and sufficient water remains in the preparation subsequent to dehydration.

Also provided herein is a pharmaceutical composition comprising the liposome of this invention and a pharmaceutically acceptable carrier. Further provided is a method of administering a compound to an animal which comprises administering to the animal the composition. The method can be used to treat an animal afflicted with a cancer, wherein a dose of the composition is administered and wherein the dose comprises an anticancer effective amount of the liposome. Typically, the dose comprises at least about 1 mg of the liposome per kg of body weight of the animal. Generally, the dose comprises from about 1 mg per kg to about 1000 mg per kg.

Provided herein is a liposome having a bilayer which comprises a lipid and a compound having the formula  $R^1-Y^1-CHZ^1-CH(NY^2Y^3)-CH_2-Z^2$ , wherein:  $R^1$  is a straight-chained alkyl, alkenyl or alkynyl group having from 5 to 19 carbon atoms in the chain;  $Y^1$  is  $-CH=CH-$ ,  $-C\equiv C-$  or  $-CH(OH)CH(OH)-$ ; each of  $Z^1$  and  $Z^2$  is independently OH or a conversion-inhibiting group; 5  $Y^2$  is H, a phenyl group, an alkyl-substituted phenyl group having from 1 to 6 carbons or an alkyl chain having from 1 to 6 carbons;  $Y^3$  is H or a group having the formula  $-R^2$ ,  $-C(O)R^2$  or  $-S(O)_2R^2$ ;  $R^2$  is a straight-chained alkyl, alkenyl or alkynyl group having from 1 to 23 carbon atoms; and wherein the bilayer comprises an anticancer-effective amount of the compound.

Also provided is a pharmaceutical composition comprising this liposome and a pharmaceutically 10 acceptable carrier. Further provided is a method of treating an animal afflicted with a cancer which comprises administering to the animal this composition.

**EXAMPLES****Example 1****Liposome Preparation**

- 5           Liposomes were prepared with the components, and at the mole ratios of components, indicated in Table 1 (see below) by the solvent evaporation method. For example, PC/Chol/C2-ceramide liposomes were prepared by dissolving 1.8242 mg bovine phosphatidylcholine (BPC), 0.4639 mg cholesterol (Chol) and 0.1366 mg C2-ceramide (C2) in a chloroform/methanol solvent mixture (2:1, volume/volume). The solvent was then evaporated to produce dried lipid,
- 10   and the dried lipid was rehydrated with HEPES buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.4). For vitamin D3-containing preparations, 0.0154 mg vitamin D3 was added to the lipid mixture. For C6-ceramide-containing preparations, 0.1590 mg C6 ceramide (C6) was substituted for the C2 ceramide. For sphingomyelin (SM)-containing preparations, 2.0470 mg SM, 0.4639 mg cholesterol and 0.0154 mg vitamin D3 were used. Furthermore, the PC/Chol
- 15   and PC/Chol/D3 preparations were prepared with 2.1280 mg BPC, 0.4639 mg cholesterol and 0.0154 mg vitamin D3.

TABLE 1

## LIPOSOME PREPARATION

<u>COMPONENTS</u>	<u>MOLAR RATIO</u>
PC:Chol:C2	6:3:1
PC:Chol:C2:D3	6:3:1:0.1
PC:Chol:C6	6:3:1
PC:Chol:C6:D3	6:3:1:0.1
SM:Chol	7:3
SM:Chol:D3	7:3:0.1
PC:Chol	7:3
PC:Chol:D3	7:3:1:0.1

PC: phosphatidylcholine; Chol: cholesterol; C2: C2 ceramide;  
D3: vitamin D3; C6: C6 ceramide; SM: sphingomyelin.

## Example 2

Effect of Various Liposomal Ceramide/Sphingomyelin Formulations on the Growth of HL-60 Cells

2 x 10<sup>5</sup> HL-60 cells were incubated with egg phosphatidylcholine/cholesterol (EPC/Chol), EPC/Chol/C2-ceramide (C2), EPC/Chol/Vitamin D3 (D3), EPC/Chol/D3/C2, EPC/Chol/C6-ceramide (C6), EPC/Chol/D3/C6, sphingomyelin (SM)/Chol and SM/Chol/D3 liposomes, as well as with buffer (no liposomes; "control") and with egg phosphatidylcholine/cholesterol (EPC/Chol) liposomes. Incubation was at 37 degrees C in serum-free medium, supplemented with 5 mg/L insulin and 5 mg/L transferrin, for 24 hours. Fetal calf serum was then added to the culture medium to a final concentration of 10%; the cells were then incubated for another 24 hours, after which the number of viable cells in each culture were counted using trypan blue staining and a hemocytometer. The number of viable cells was determined for lipid doses of 100  $\mu$ M and 200 $\mu$ M, and is given in the figures (below) as the number of viable cells per ml of culture medium, times 10,000. Results are reported in Figure 3 and Table 2 (see below).

**Example 3**Effect of Various Liposomal Ceramide/Sphingomyelin Formulations on the Growth of P388 Cells

5         $2 \times 10^5$  P388 cells were incubated with various ceramide or sphingomyelin liposomal formulations (see Example 2, above), as well as with buffer alone and with egg phosphatidylcholine/cholesterol (EPC/Chol) liposomes, under the conditions given above. The number of viable cells in the cultures was determined for lipid doses of 50  $\mu$ M, 100  $\mu$ M and 200  $\mu$ M. Results are reported in Figure 4 and Tables 2 and 3.

10

**Example 4**15    Effect of Various Liposomal Ceramide/Sphingomyelin Formulations on the Growth of U937 Cells

$2 \times 10^5$  U937 cells were incubated with the various ceramide or sphingomyelin liposomal formulations indicated above (see Example 2), as well as with buffer alone and with egg phosphatidylcholine/cholesterol (EPC/Chol) liposomes, under the conditions given above. The number of viable cells in the cultures was determined for lipid doses of 50  $\mu$ M, 100  $\mu$ M and 200  $\mu$ M. Results are reported in Figures 5 and 7, and Tables 2 and 3.

20

**Example 5**25    Effect of Various Liposomal Ceramide/Sphingomyelin Formulations on the Growth of RPMI-7666 Cells

$2 \times 10^5$  RPMI-7666 cells were incubated with the various ceramide/sphingomyelin liposomal formulations indicated above (see example 2), as well as with no liposomes (control) and with egg phosphatidylcholine/cholesterol (EPC/Chol) liposomes, under the conditions indicated above. The number of viable cells in the cultures was determined. Results are reported in Figures 6 and 7, and Tables 2 and 3.

30

**Example 6**Effect of Various Liposomal Ceramide/Sphingomyelin Formulations on the Growth of CHO/K15 Cells

2 x 10<sup>5</sup> CHO/k1 cells were incubated with the various ceramide/sphingomyelin liposomal formulations indicated above (see example 2), as well as with no liposomes (control) and with egg phosphatidylcholine/cholesterol (EPC/Chol) liposomes, under the conditions indicated above. The number of viable cells in the cultures was determined. Results are  
10 reported in Table 2.



TABLE 2

Survival of Various Cancer Lines (Without Serum)

% Survival (By trypan blue exclusion assay)					
Formulations	RPMI-7666	U-937	P-388	HL-60	CHO/k1
BPC/CHOL/ VD3	129.7, 107.1	113.4, 130	136, 103	87	138
Control	100	100	100	100	100
BPC/CHOL/VD 3/C-6	103, 90	63.5, 55	49.6, 26	37	73
FREE C-6 CERAMIDE	79	82.6	55.4	-	-
FREE C6 m- silyl-ester	85.6	80.6	-	-	-
BPC/CHOL/VD 3/C-2	93.7	49	46	47	90

5

Bioactive lipid dose = 20 uM

TABLE 3

Effect of Free and Liposomal ceramide on various cancer lines (With serum)

% Survival (By trypan blue assay)			
Formulations	RPMI-7666	U-937	P-388
Control	100	100	100
BPC/CHOL/VD3/C-6	93.8 82 (thy)*	84.3 75.1(thy)*	55.0 84.7(thy)*
FREE C-6 CERAMIDE	89.2	97	84.5

Bioactive lipid dose = 20  $\mu$ M; \*: growth inhibition measured by standard thymidine incorporation assay.

#### Example 7

##### Therapeutic Efficacy of Liposomal Ceramides in Mice

CDF1 mice were each injected intraperitoneally with  $2.5 \times 10^6$  P388 cells. Groups having ten mice each were intraperitoneally administered either a HEPES-buffered saline control (10 mM HEPES, 150 mM NaCl, pH 7.4), or liposomal vitamin D3, liposomes containing C2 ceramide or liposomes containing C6 ceramide, prepared in accordance with the procedures described in Example 1 (see above), at a lipid dose of 1.5 mg of lipid per kg of body weight of the mice, the administration being 24 hours administration of the p388 cells. Survival was assessed at various times post-liposome/control administration. Results are presented in Figure 8.

**Example 8****5    In Vitro Cytotoxicity Studies**

These studies were performed using a sulforrhodamine B assay (see Monks et al., J. Natl. Cancer Inst. (U.S.) 83: 757 (1987)). Compounds were dissolved in ethanol. Results from these studies, presented in the following tables, are expressed as GI<sub>50</sub> values, that is,  
10    concentration of a drug (micromolar) required to inhibit growth of fifty percent of the cells.

Table 4

*In Vitro* Drug Sensitivity of Human and Mouse Cell Lines to  
Ceramide Derivatives

72 Hour Drug Exposure [GI<sub>50</sub> (uM)  $\pm$  SD]

Ceramide and derivatives	C6-Ceramide	1-Acetate C6-Ceramide	1,3-Diacetate C6-Ceramide	1-Butyrate C6-Ceramide	1-Isobutyrate C6-Ceramide
A549	12.0 $\pm$ 4.8	12.7 $\pm$ 7.6	6.1 $\pm$ 1.1	6.2 $\pm$ 0.4	7.9 $\pm$ 3.5
MCF7	24.2 $\pm$ 8.5	ND	15.5 $\pm$ 0.2	17.9 $\pm$ 10.0	ND
MCF7/ADR	36.025 $\pm$ 0.88	ND	42.89 $\pm$ 3.6	37.80 $\pm$ 8.06	37.80 $\pm$ 4.67
RPMI 7666	8.5 $\pm$ 3.5	9.0 $\pm$ 2.0	ND	ND	ND
U937	7.0 $\pm$ 1.7	11.3 $\pm$ 1.0	ND	ND	ND
LEWIS LUNG	9.6 $\pm$ 4.4	8.5 $\pm$ 0.4	4.9 $\pm$ 0.5	6.4 $\pm$ 2.4	ND

ND-Not Done;

\* One Experiment Only

**Table 5**

*In Vitro* Drug Sensitivity of Human and Mouse Cell Lines to  
Ceramide Derivatives  
72 Hour Drug Exposure [GI<sub>50</sub> (uM)  $\pm$  SD]

Ceramide and Derivatives	C6-Ceramide	1-TBDMS C6-Ceramide	3-TBDMS C6-Ceramide	1,3-Di-TBDMS C6-Ceramide	1-TBDMS-3-Butyrate C6-Cer
A549	12.0 $\pm$ 4.8	>200 *	>200 *	>200	>200 *
RPMI 7666	8.5 $\pm$ 3.5	>200 *	>200 *	>200	>200 *
U937	7.0 $\pm$ 1.7	>200 *	>200 *	134.5 $\pm$ 28.8	>200 *
C3H10T1/2	14.0 $\pm$ 2.5	>161.2 *	>200 *	ND	>200 *
LEWIS LUNG	9.6 $\pm$ 4.4	>200 *	>200 *	>200	>200 *

ND-Not Done; \* One Experiment Only

# TABLE 6

## *In Vitro* Drug Sensitivity of Human and Mouse Cell Lines to Ceramide Derivatives

72 Hour Drug Exposure [  $GI_{50}(uM) \pm SD$  ]

Ceramide and Derivatives	C6-Ceramide	Sphingosine	N-Hexyl Sphingosine	1-Acetate-3-one C6-Cer	4,5-Diol C6-Ceramide
A549	12.0 $\pm$ 4.8	20.2 $\pm$ 0.8 *	4.9 $\pm$ 0.1	14.4 $\pm$ 0.1 *	25.1 $\pm$ 0.3 *
MCF7	24.2 $\pm$ 8.5	20.4 $\pm$ 0.4 *	4.8 $\pm$ 0.1	6.7 $\pm$ 0.1 *	20.7 $\pm$ 0.1 *
MCF7/ADR	36.03 $\pm$ 0.88	19.8 $\pm$ 0.0 *	5.57 $\pm$ 1.17	14.7 $\pm$ 0.1 *	28.3 $\pm$ 1.1 *
CAKI 1	6.04 $\pm$ 0.23	39.7 $\pm$ 0.8 *	6.84 $\pm$ 3.62	ND	ND
OVCAR 3	15.15 $\pm$ 1.77	44.6 $\pm$ 0.9 *	4.99 $\pm$ 0.05 *	15.3 $\pm$ 0.1 *	38.2 $\pm$ 2.3 *
HT 29	4.0 $\pm$ 0.2	19.3 $\pm$ 0.1 *	5.2 $\pm$ 0.1 *	13.9 $\pm$ 0.2 *	14.6 $\pm$ 0.4 *
SKMEL 28	13.3 $\pm$ 2.51	15.6 $\pm$ 1.0 *	4.94 $\pm$ 0.66 *	ND	ND
P388	6.24 $\pm$ 0.3	ND	2.59 $\pm$ 0.3 *	ND	ND
P388/ADR	12.7 $\pm$ 1.7 *	ND	2.61 $\pm$ 1.7 *	ND	ND
LEWIS LUNG	9.6 $\pm$ 4.4	12.2 $\pm$ 0.1	4.9 $\pm$ 0.1	14.5 $\pm$ 0.1	15.2 $\pm$ 0.1

ND-Not Done;

\* One Experiment Only

TABLE 7

*In Vitro* Drug Sensitivity of Selected Sphingosine Derivatives on a Diverse  
Tumor Cell Line Panel

## 72 Hour Drug Exposure

Cell Lines	N-C4 Sphingosine	N-C6 Sphingosine	N-C8 Sphingosine	N-C8 Sphingosine HCl Salt	N-C10 Sphingosine	Cer-C6
A549	4.90+/-0.10	4.90+/-0.06	4.91+/-0.01	4.97+/-0.19	4.99+/-0.08	8.08+/-0.06
MCF 7	4.88+/-0.03	4.74+/-0.09	4.88+/-0.11	4.90+/-0.02	5.13+/-0.01	12.70+/-0.14
MCF 7/ADR	9.60+/-0.47	4.69+/-0.10	4.88+/-0.04	4.82+/-0.18	6.19+/-0.43	26.1+/-0.85
OVCAR 3	14.05+/-0.35	4.99+/-0.05	5.41+/-0.13	5.23+/-0.01	12.50+/-0.85	15.15+/-1.77
CAKI 1	6.88+/-0.52	4.28+/-0.04	4.64+/-0.07	4.47+/-0.01	6.18+/-0.01	5.88+/-0.10
SKMEL 28	6.13+/-1.03	4.94+/-0.06	4.96+/-0.03	4.95+/-0.02	13.00+/-0.00	11.55+/-0.07
HT 29	6.49+/-0.11	5.03+/-0.06	5.72+/-0.24	5.78+/-0.00	14.50+/-0.14	4.67+/-0.19
LEWIS LUNG	5.08+/-0.13	4.61+/-0.04	5.05+/-0.02	4.82+/-0.18	6.71+/-1.04	5.81+/-0.31

GI50 (uM)+/- SD

**TABLE 8**  
***In Vitro* Sensitivity of A549 and MCF 7/ADR to C6-Ceramide Derivatives at  
 Different Drug Exposure Times**

**72 Hour Post-Drug Addition Incubation**

Drug Exposure Time	A549					MCF 7/ADR			
	1-Acetate C6-Ceramide	1-Butyrate C6-Ceramide	1-Isobutyrate C6-Ceramide	N-C8 Sphingosine		1-Acetate C6-Ceramide	1-Butyrate C6-Ceramide	1-Isobutyrate C6-Ceramide	N-C8 Sphingosine
1 Hour Pulse	92.9+/-1.3	>100	>100	31.2+/-5.0		>100	>100	>100	34.3+/-4.2
4 Hour Pulse	44.1+/-1.3	52.9+/-2.5	>100	12.8+/-1.0		59.5+/-1.4	79.1+/-5.3	>100	14.2+/-1.2
8 Hour Pulse	35.3+/-2.5	36.5+/-0.8	40.2+/-2.5	6.7+/-0.2		42.7+/-1.2	44.0+/-1.1	52.3+/-1.6	8.9+/-0.2
24 Hour Pulse	7.2+/-0.2	8.6+/-0.6	8.4+/-0.7	5.0+/-0.1		19.5+/-0.8	20.8+/-1.8	22.7+/-1.1	4.8+/-0.1
48 Hour Pulse	4.35+/-0.1	4.7+/-0.1	5.2+/-0.3	4.8+/-0.1		15.6+/-0.9	17.2+/-0.1	18.7+/-0.5	4.62+/-0.1
72 Hour Continuous Exposure	5.1+/-0.1	5.5+/-0.2	5.6+/-0.2	5.0+/-0.3		16.7+/-1.7	18.5+/-0.4	19.6+/-1.0	5.0+/-0.1

GI<sub>50</sub> (uM) +/- SD



## Example 9

In Vivo Toxicity Studies

- 5 These studies were performed by intravenous injection of n-hexyl sphingosine, at the indicated dose (see below), into mice. Results are presented below.

**Table 9**  
**Toxicity of N-hexyl sphingosine after i.v injection in mice**

<b>Dose (mg/kg)</b>	<b>Number of Animals Dead</b>
<b>Control (Tween-80)</b>	<b>0/2</b>
<b>100</b>	<b>2/2</b>
<b>50</b>	<b>0/2*</b>
<b>25</b>	<b>0/2**</b>
<b>12.5</b>	<b>0/2</b>

**\* Immediate ataxia, decreased activity. Respiration: Gasping.**

- **After 30 minute decreased activity was still observed.**
- **After 24 hours no abnormality was observed.**

**\*\* Decrease activity immediately after injection.**

- **After 30 minute no abnormality was observed.**

**Example 10***In Vivo Studies*

- 5           These studies were performed using P-388/adriamycin resistant leukemia cells. Mice were injected i.p. with 100,000 cells, and then treated on days 1, 3 and 5 post-injection with n-hexyl sphingosine. Results are presented in Figure 14.

**Example 11**Compound Synthesis

- 5    **Synthesis of silyl ether of ceramide:** The mixture of Ceramide and t-Butyldimethylsilyl chloride ( 1 equivalent) and imidazole ( 2 equivalent) in DMF was stirred under N<sub>2</sub> at room temperature overnight. The solvent was removed under a stream of N<sub>2</sub> and residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub>, washed (H<sub>2</sub>O), dried (MgSO<sub>4</sub>) and concentrated to dryness. The residue was purified over silica gel (AcOEt: Hexan=1:3).
- 10    **Synthesis of 1-ester ceramide:** The mixture of ceramide and Ac<sub>2</sub>O ( 1 equivalent) and catalytic amount of dimethyl amino pyridine in dry CH<sub>2</sub>Cl<sub>2</sub> was stirred at room temperature for 1 hour and the reaction was checked by TLC (AcOEt). The mixture was concentrated. The crude product was purified over silica gel (AcOEt: Hexane = 2:3.5) .
- 15    **Oxidation of C3-OH of ceramide to ketone:** 1-OAc ceramide was dissolved in acetone and cooled in ice-bath. Jones's reagent was dropwised slowly till the orange color persistent. The reaction was quenched by isopropanol, and NaHCO<sub>3</sub> was added and stirred for 5 minutes. The solution was filtrate and concentrated to dryness. The crude product was purified by
- 20    preparative TLC (ACOET: Hexane = 1:2.5).
- 25    **Reduction of ceramide to sphingosine analogs:** To an ice-cold stirred solution of ceramide in anhydrous THF was added LiAlH<sub>4</sub> and the mixture was stirred at room temperature under N<sub>2</sub> for 24 hours. Under ice cooling the reaction mixtures was quenched by addition of saturated aqueous NaHCO<sub>3</sub>. The resulting slurry was filtered and washed with THF. The solution was concentrated and the residue was brought into CH<sub>2</sub>Cl<sub>2</sub>, washed with H<sub>2</sub>O, dried (MgSO<sub>4</sub>) and concentrated to dryness. The residue was purified over preparative TLC (silica gel) CH<sub>2</sub>Cl<sub>2</sub> : MeOH : TEA=8 :1 : 0.08.
- 30    **Synthesis of 4,5-diol ceramide:** To a solution of ceramide in a mixture of Me<sub>2</sub>CO distilled H<sub>2</sub>O and t-BuOH, N-Methyl morpholine N-oxide (NMO, 1.2 equivalent) and OsO<sub>4</sub> (catalytic amount) in THF were added. The reaction mixture was stirred at 45°C for 6 hours and it was quenched by solid NaHCO<sub>3</sub> and the mixture was stirred for 15 minutes. The suspension was filtered and filtrate was dissolved in THF. The solution was washed with brine. The organic
- 35    solution was separated , dried and concentrated to dryness. The residue was purified over preparative TLC (THF).

What is claimed is:

1. A compound having the formula  $R^1-Y^1-CHZ^1-CH(NY^2Y^3)-CH_2-Z^2$ , wherein:
  - $R^1$  is a straight-chained alkyl, alkenyl or alkynyl group having from 8 to 19 carbon atoms in the aliphatic chain;
  - $Y^1$  is  $-CH=CH-$ ,  $-C\equiv C-$  or  $-CH(OH)CH(OH)-$ ;
  - $Z^1$  is OH or a conversion-inhibiting group;
  - $Z^2$  is a conversion-inhibiting group;
  - $Y^2$  is H, a phenyl group, an alkyl-substituted phenyl group having from 1 to about 6 carbons in the alkyl chain, or an alkyl chain having from 1 to 6 carbons;
  - $Y^3$  is H or a group having the formula  $-C(O)R^2$  or  $-S(O)_2R^2$ ;
  - $R^2$  is a straight-chained alkyl, alkenyl or alkynyl group having from 1 to 23 carbon atoms in the chain;
  - and when  $Z^2$  is an amino,  $R^2$  is an aliphatic chain having from 1 to 9 or from 19 to 23 carbon atoms in the aliphatic chain.
2. The compound of claim 1, wherein  $R^1$  is an alkyl group.
3. The compound of claim 2, wherein the alkyl group is  $CH_3(CH_2)_{12}-$ .
4. The compound of claim 1, wherein  $Y^1$  is  $-CH=CH-$ .
5. The compound of claim 1, wherein  $Y^2$  is H.
6. The compound of claim 1, wherein  $Y^3$  is  $-C(O)R^2$ .
7. The compound of claim 6, wherein  $R^2$  is an alkyl chain.
8. The compound of claim 1, wherein:
  - the conversion-inhibiting group is a group having the formula  $-X^2X^3$  or  $-O-X^2X^3$ ;

$X^2$  is selected from the group consisting of  $CH_2-$ ,  $C(CH_3)_2-$ ,  $Si(PO_4)_2-$ ,  $Si(CH_3)_2-$ ,  $SiCH_3PO_4-$ ,  $C(O)-$  and  $S(O)_2-$  and

5  $X^3$  is selected from the group consisting of  $-C(O)H$ ,  $-CO_2H$ ,  $-CH_3$ ,  $-C(CH_3)_3$ ,  $-Si(CH_3)_3$ ,  $-SiCH_3(C(CH_3)_3)_2$ ,  $-Si(C(CH_3)_3)_3$ ,  $-Si(PO_4)_2C(CH_3)_3$ , a phenyl group, an alkyl-substituted phenyl group having from 1 to 6 carbons in the alkyl chain, an alkyl chain having from 1 to 6 carbons, an amino moiety, a chlorine, a fluorine, or a group having the formula  $C(R^3R^4)OH$ , wherein each of  $R^3$  and  
10  $R^4$  is independently an alkyl chain having from 1 to 6 carbons, a phenyl group or an alkyl-substituted phenyl group having from 1 to 6 carbons in the alkyl chain.

9. The compound of claim 8, wherein the conversion-inhibiting group is  $-OC(O)CH_3$ ,  $-OC(O)CH_2CH_2CH_3$ ,  $-OC(O)CH(CH_3)CH_3$ , or  $-OSi(CH_3)_2C(CH_3)_3$ .  
15

10. The compound of claim 9, wherein the conversion-inhibiting group is  $-OSi(CH_3)_2C(CH_3)_3$ .

20 11. The compound of claim 1, wherein:  
the conversion-inhibiting group is a group having the formula  $-X^1$  or  $-OX^1$ ;  
 $X^1$  is  $C(O)H$ ,  $CO_2H$ ,  $CH_3$ ,  $C(CH_3)_3$ ,  $Si(CH_3)_3$ ,  $SiCH_3(C(CH_3)_3)_2$ ,  
 $Si(C(CH_3)_3)_3$ ,  
25

$Si(PO_4)_2C(CH_3)_3$ , a phenyl group, an alkyl-substituted phenyl group having from 1 to 6 carbons in the alkyl chain, an alkyl chain having from 1 to 6 carbons, an amino moiety, a fluorine, a chlorine, or a group having the formula  $C(R^3R^4)OH$ ; and  
30

each of  $R^3$  and  $R^4$  is independently an alkyl chain having from 1 to 6 carbons.

12. The compound of claim 1 having the formula  $CH_3(CH_2)_{12}-CH=CH-CH_2Z^1-CH(NHY^3)-CH_2-Z^2$ .  
35

13. The compound of claim 12, wherein  $Y^3$  is a group having the formula  $-C(O)R^2$ .

14. The compound of claim 13, wherein  $Y^3$  is  $-C(O)(CH_2)_4CH_3$ .
15. The compound of claim 12, wherein  $Z^2$  is  $-OSi(CH_3)_2C(CH_3)_3$ ,  $-OSi(PO_4)_2C(CH_3)_3$ ,  $-C(O)CH_3$  or  $-OC(O)CH_2CH_2CH_3$ .
- 5 16. A pharmaceutical composition comprising the compound of claim 1 and a pharmaceutically acceptable carrier.
17. The composition of claim 16 comprising an additional bioactive agent.
- 10 18. A method of administering a bioactive compound to an animal which comprises administering to the animal the composition of claim 16.
19. The method of claim 18, wherein the animal is a human.
- 15 20. The method of claim 18, wherein the animal is afflicted with a cancer and wherein the composition comprises an anticancer effective amount of the compound.
21. The method of claim 20, wherein the anticancer effective amount of the compound is at least about 0.1 mg of the compound per kg of body weight of the animal.
- 20 22. The method of claim 21, wherein the anticancer effective amount is from about 1 mg per kg to about 50 mg per kg.
- 25 23. The method of claim 20, wherein the cancer is a brain, breast, lung, ovarian, colon, stomach or prostate cancer.
24. The method of claim 20, wherein the cancer is a sarcoma, carcinoma, neuroblastoma, or glioma.
- 30 25. The method of claim 20, wherein the cancer is a drug resistant cancer.
26. The method of claim 18, comprising administering an additional bioactive agent to the animal.
- 35 27. A liposome having a bilayer which comprises a lipid and a compound having the formula  $R^1-Y^1-CHZ^1-CH(NY^2Y^3)-CH_2-Z^2$ , wherein:

37

$R^1$  is a straight-chained alkyl, alkenyl or alkynyl group having from 5 to 19 carbon atoms in the chain;

$Y^1$  is  $-CH=CH-$ ,  $-C\equiv C-$  or  $-CH(OH)CH(OH)-$ ;

5

each of  $Z^1$  and  $Z^2$  is independently OH or a conversion-inhibiting group;

$Y^2$  is H, a phenyl group, an alkyl-substituted phenyl group having from 1 to 6 carbons in the alkyl chain, or an alkyl chain having from 1 to 6 carbons;

10

$Y^3$  is H or a group having the formula  $-R^2$ ,  $-C(O)R^2$  or  $-S(O)_2R^2$ ;

$R^2$  is a straight-chained alkyl, alkenyl or alkynyl group having from 1 to 23 carbon atoms;

15

and wherein the bilayer comprises at least about five mole percent of the compound.

28. The liposome of claim 27, wherein  $Y^3$  is a group having the formula  $R^2$ .
29. The liposome of claim 28, wherein  $R^2$  is  $-(CH_2)_3CH_3$ ,  $-(CH_2)_5CH_3$ ,  $-(CH_2)_7CH_3$ , or  $-(CH_2)_9CH_3$ .
30. The liposome of claim 29, wherein  $R^2$  is  $-(CH_2)_5CH_3$ .
31. The liposome of claim 27, wherein  $Y^3$  is a group having the formula  $-C(O)R^2$ .
32. The liposome of claim 31, wherein  $Y^3$  is  $-C(O)(CH_2)_4CH_3$ .
33. The liposome of claim 27, wherein at least one of  $Z^1$  and  $Z^2$  is a conversion-inhibiting group.
34. The liposome of claim 33, wherein the conversion-inhibiting group is  $-OC(O)CH_3$ ,  $-OC(O)CH_2CH_2CH_3$ ,  $-OC(O)CH(CH_3)CH_3$ , or  $-OSi(CH_3)_2C(CH_3)_3$ .
35. The liposome of claim 34, wherein the conversion-inhibiting group is  $-OSi(CH_3)_2C(CH_3)_3$ .

38

36. The liposome of claim 27, wherein the compound has the formula  $\text{CH}_3\text{-(CH}_2\text{)}_{12}\text{-CH=CH-CH}_2\text{Z}^1\text{-CH(NHY}^3\text{)-CH}_2\text{Z}^2$ .
37. The liposome of claim 27, wherein the bilayer comprises at least about 10 mole percent of the compound.
38. The liposome of claim 27, wherein the bilayer comprises vitamin D<sub>3</sub>.
39. The liposome of claim 38, wherein the bilayer comprises about 1 mole percent of vitamin D<sub>3</sub>.
40. The liposome of claim 27, wherein the bilayer comprises a headgroup-modified lipid.
41. The liposome of claim 27 comprising an additional bioactive agent.
42. The liposome of claim 27, wherein the liposome is dehydrated.
43. A pharmaceutical composition comprising the liposome of claim 27 and a pharmaceutically acceptable carrier.
44. A method of administering a compound to an animal which comprises administering to the animal the composition of claim 43.
45. The method of claim 44, wherein the animal is afflicted with a cancer, wherein a dose of the composition is administered and wherein the dose comprises an anticancer effective amount of the liposome.
46. The method of claim 45, wherein the dose comprises at least about 1 mg of the liposome per kg of body weight of the animal.
47. The method of claim 46, wherein the dose comprises from about 1 mg per kg to about 1000 mg per kg.
48. A liposome having a bilayer which comprises a lipid and a compound having the formula  $\text{R}^1\text{-Y}^1\text{-CHZ}^1\text{-CH(NY}^2\text{Y}^3\text{)-CH}_2\text{-Z}^2$ , wherein:  
R<sup>1</sup> is a straight-chained alkyl, alkenyl or alkynyl group having from 5 to 19 carbon atoms in the chain;



39

$Y^1$  is  $-CH=CH-$ ,  $-C\equiv C-$  or  $-CH(OH)CH(OH)-$ ;

each of  $Z^1$  and  $Z^2$  is independently OH or a conversion-inhibiting group;

5  $Y^2$  is H, a phenyl group, an alkyl-substituted phenyl group having from 1 to 6 carbons or an alkyl chain having from 1 to 6 carbons;

$Y^3$  is H or a group having the formula  $-R^2$ ,  $-C(O)R^2$  or  $-S(O)_2R^2$ ;

10  $R^2$  is a straight-chained alkyl, alkenyl or alkynyl group having from 1 to 23 carbon atoms;

and the bilayer comprises an anticancer-effective amount of the compound.

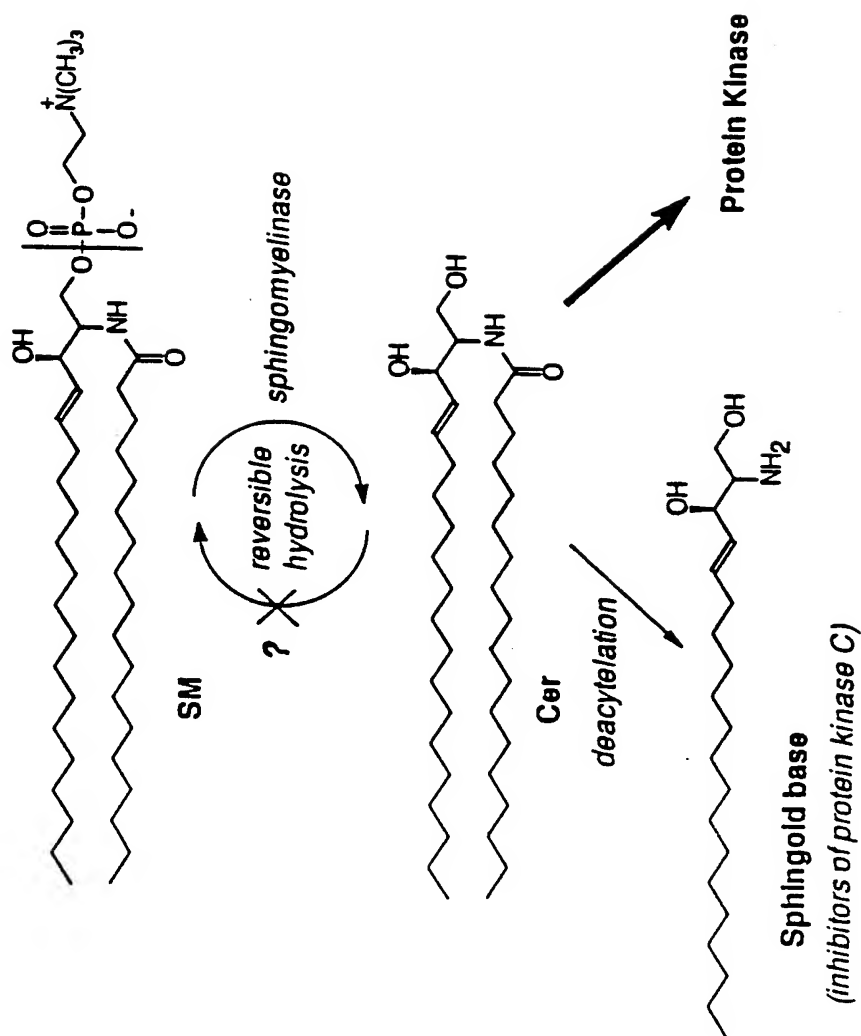
15 49. A pharmaceutical composition comprising the liposome of claim 48 and a pharmaceutically acceptable carrier.

50. A method of treating an animal afflicted with a cancer which comprises administering to the animal the composition of claim 49.

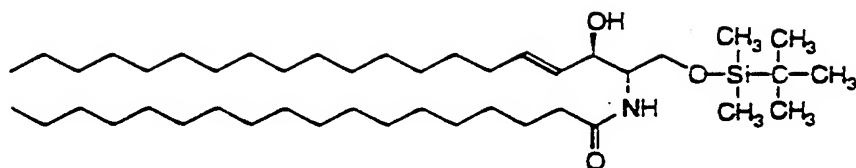
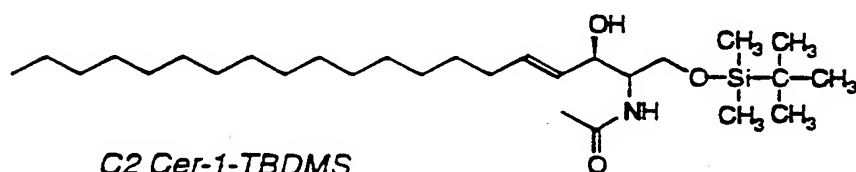
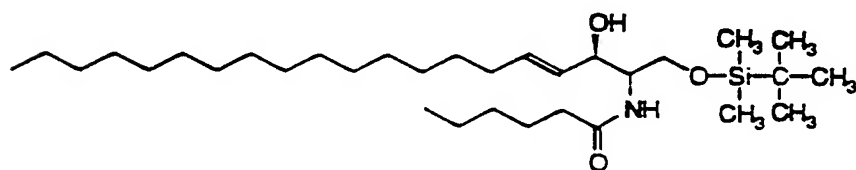
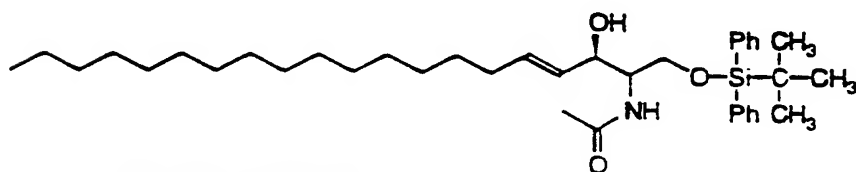
20

1/21

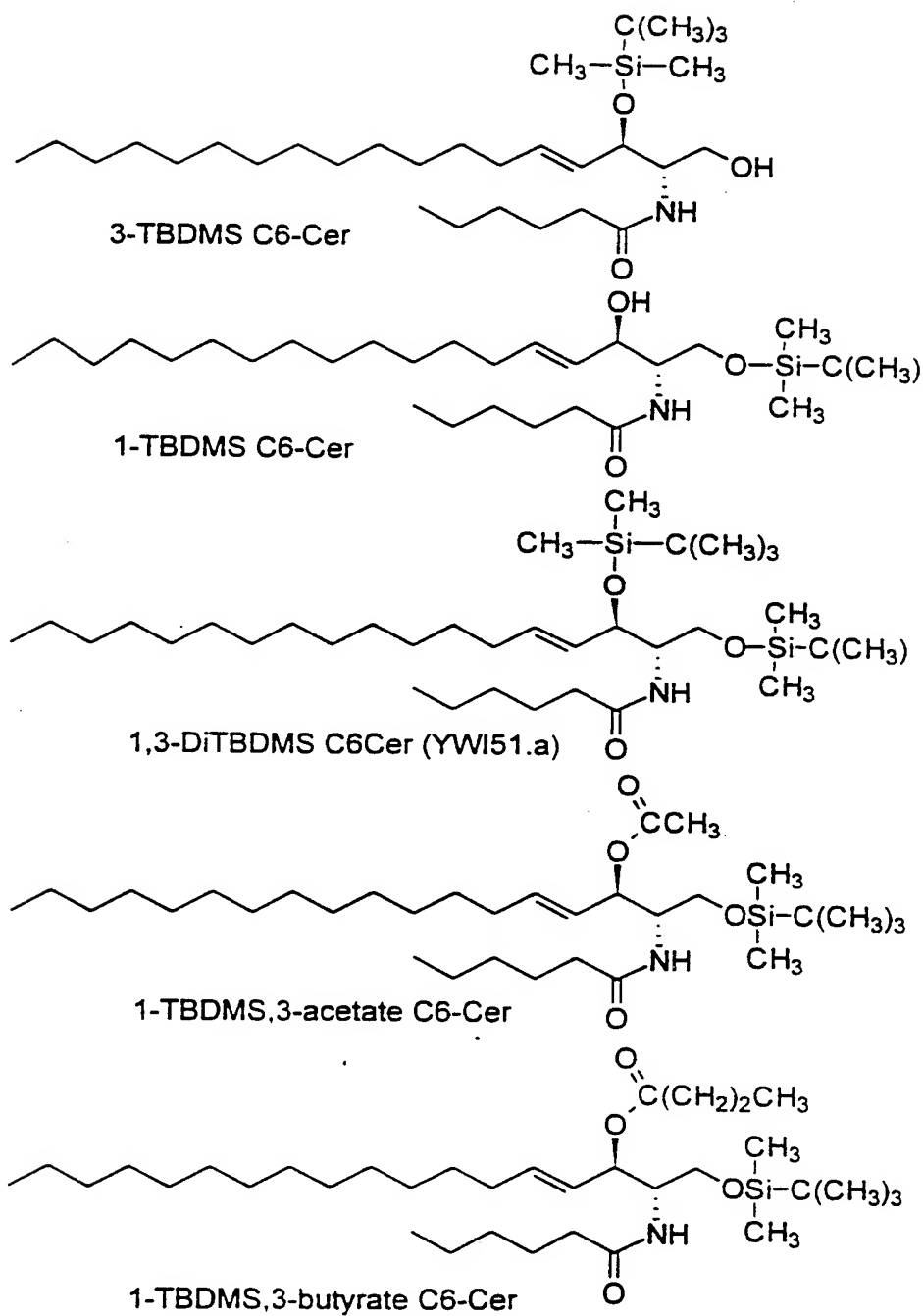
Fig. 1



2/21

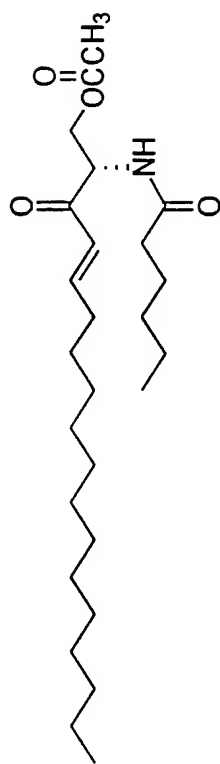
**Fig. 2a***Type III Cer-1-TBDMS**C2 Cer-1-TBDMS**C6 Cer-1-TBDMS**C2 Cer-1-TBDPS*

3/21

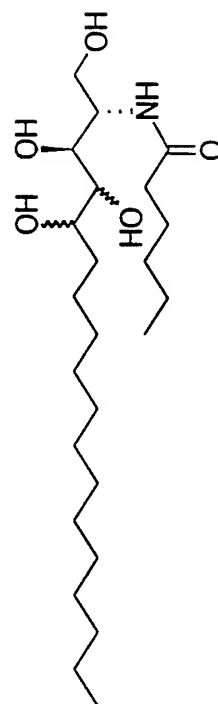
**Fig. 2b**

4/21

Fig. 2c

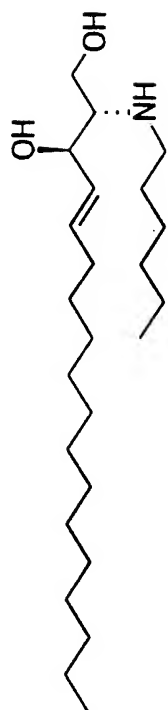
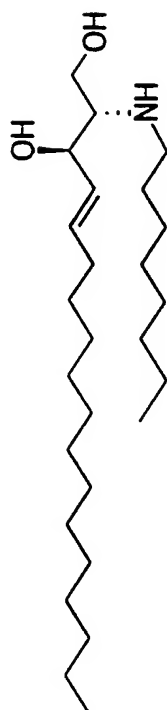
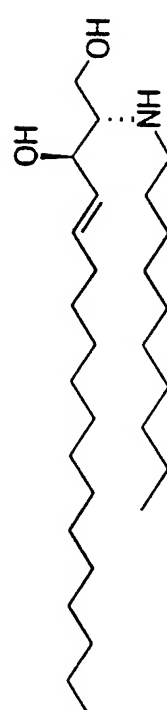


1-Acetate-3-one C6-Cer



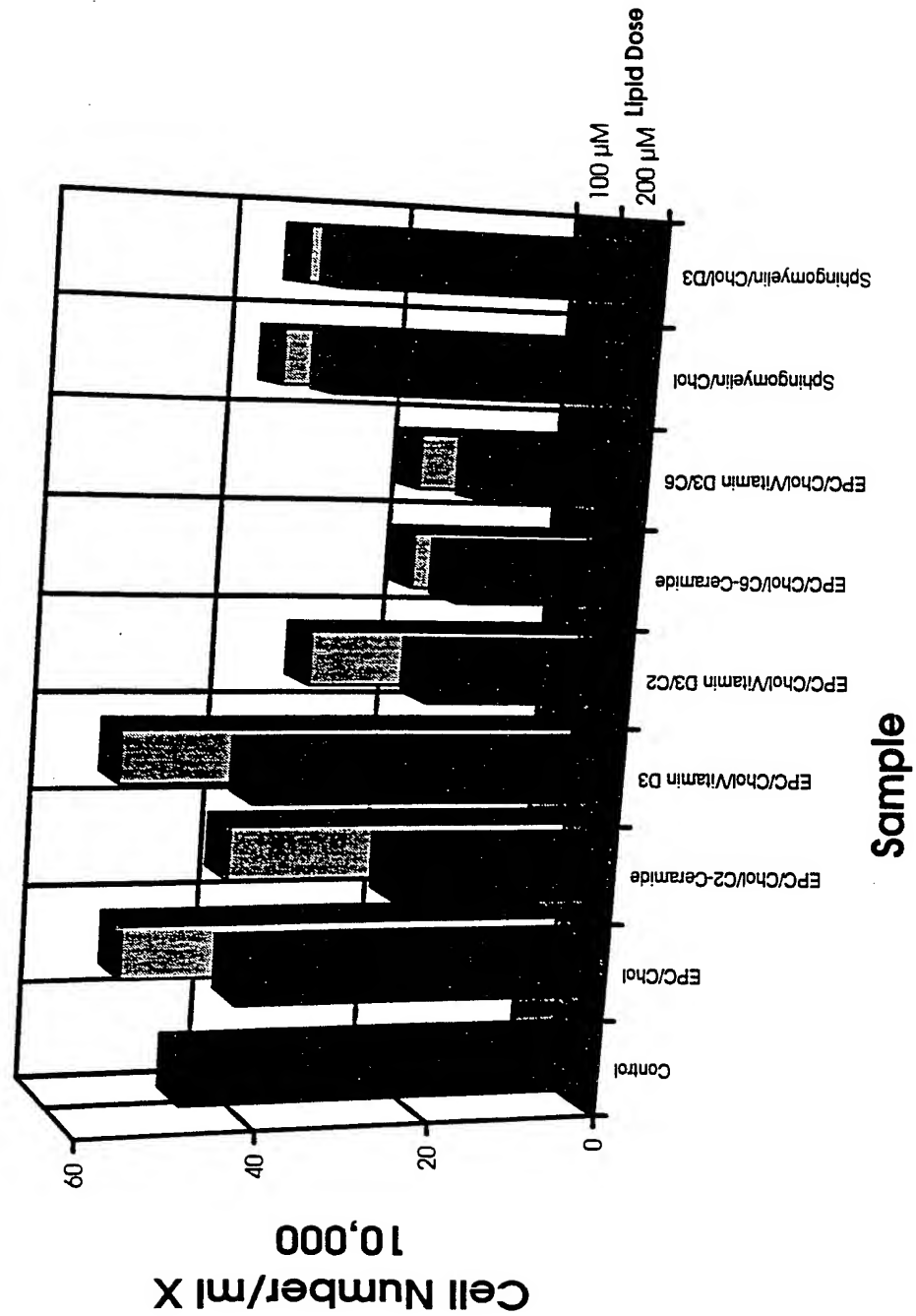
4,5-Diol C6-Cer

5/21

**Fig. 2d****N-Hexyl Sphingosine (or N-C6 Sphingosine)****N-C8 Sphingosine****N-C10 Sphingosine**

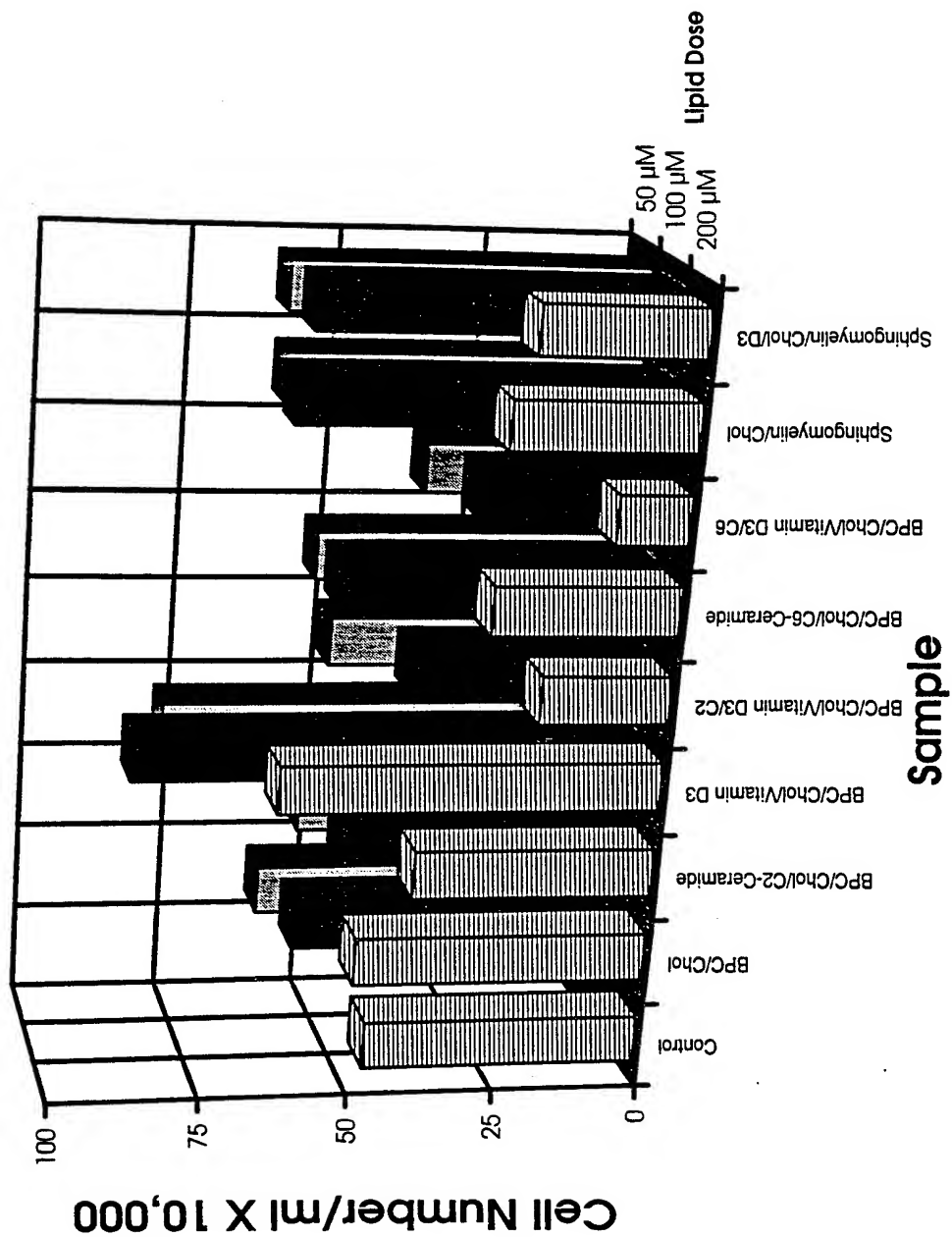
6/21

Fig. 3



7/21

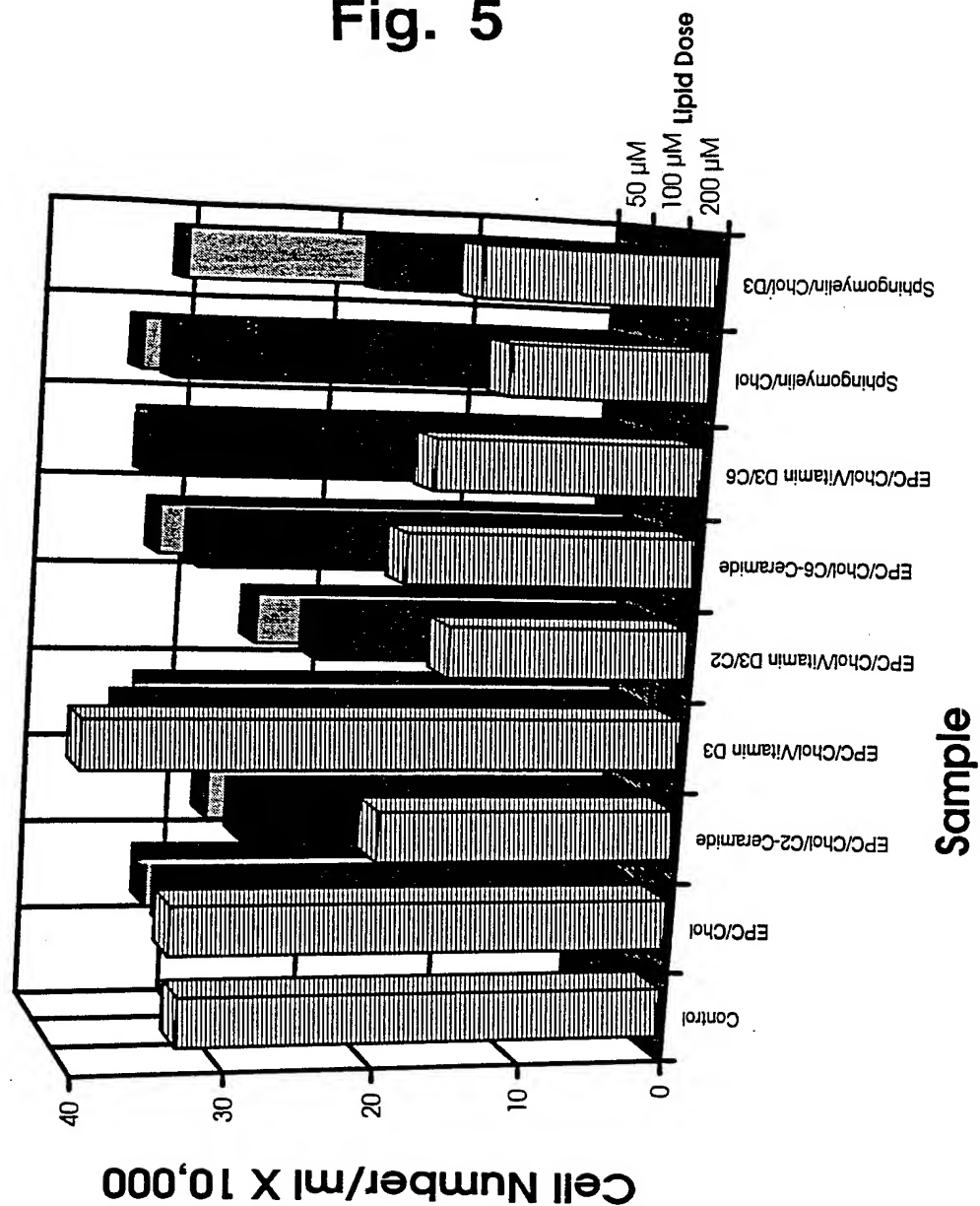
Fig. 4





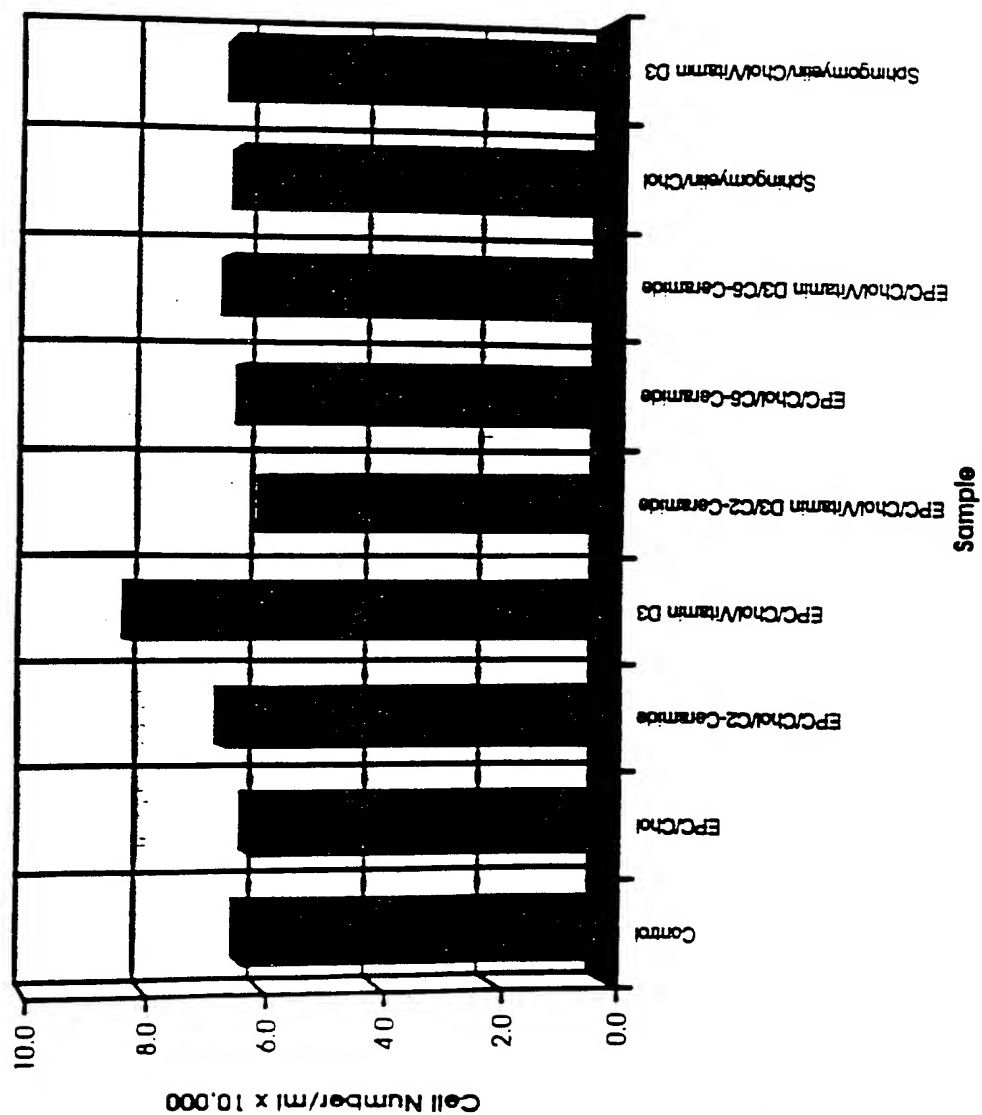
8/21

Fig. 5



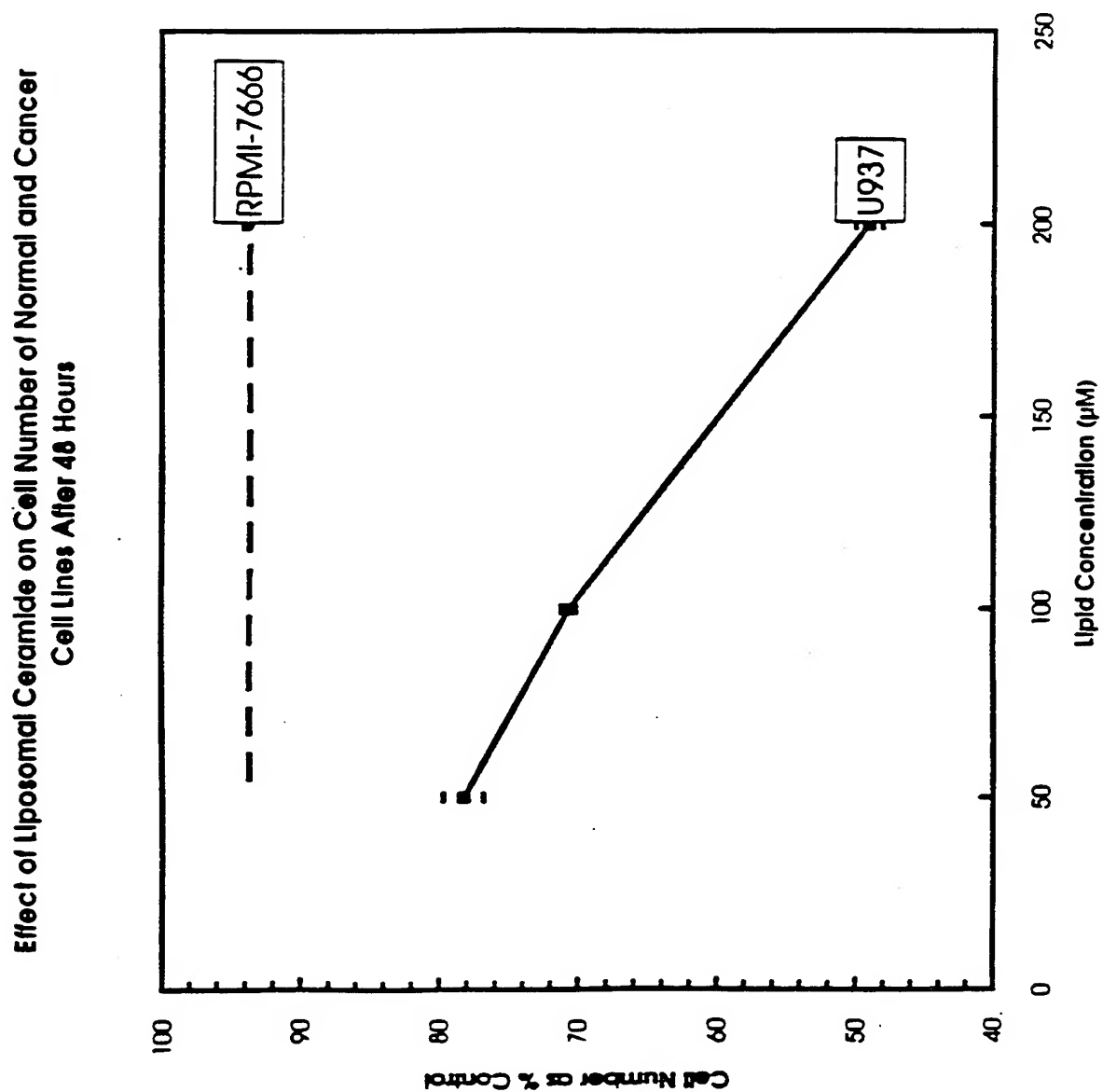
9/21

Fig. 6



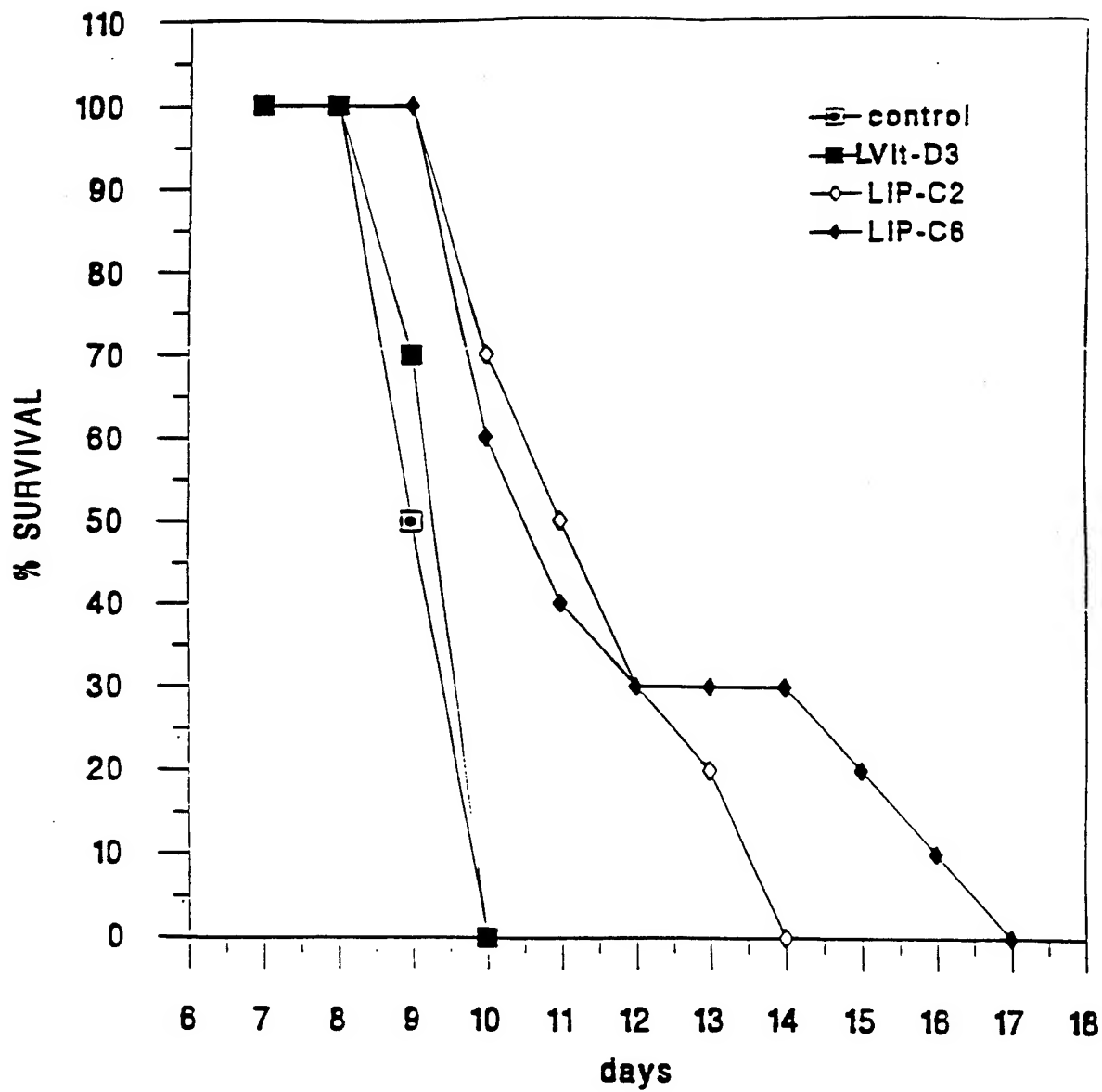
10/21

Fig. 7



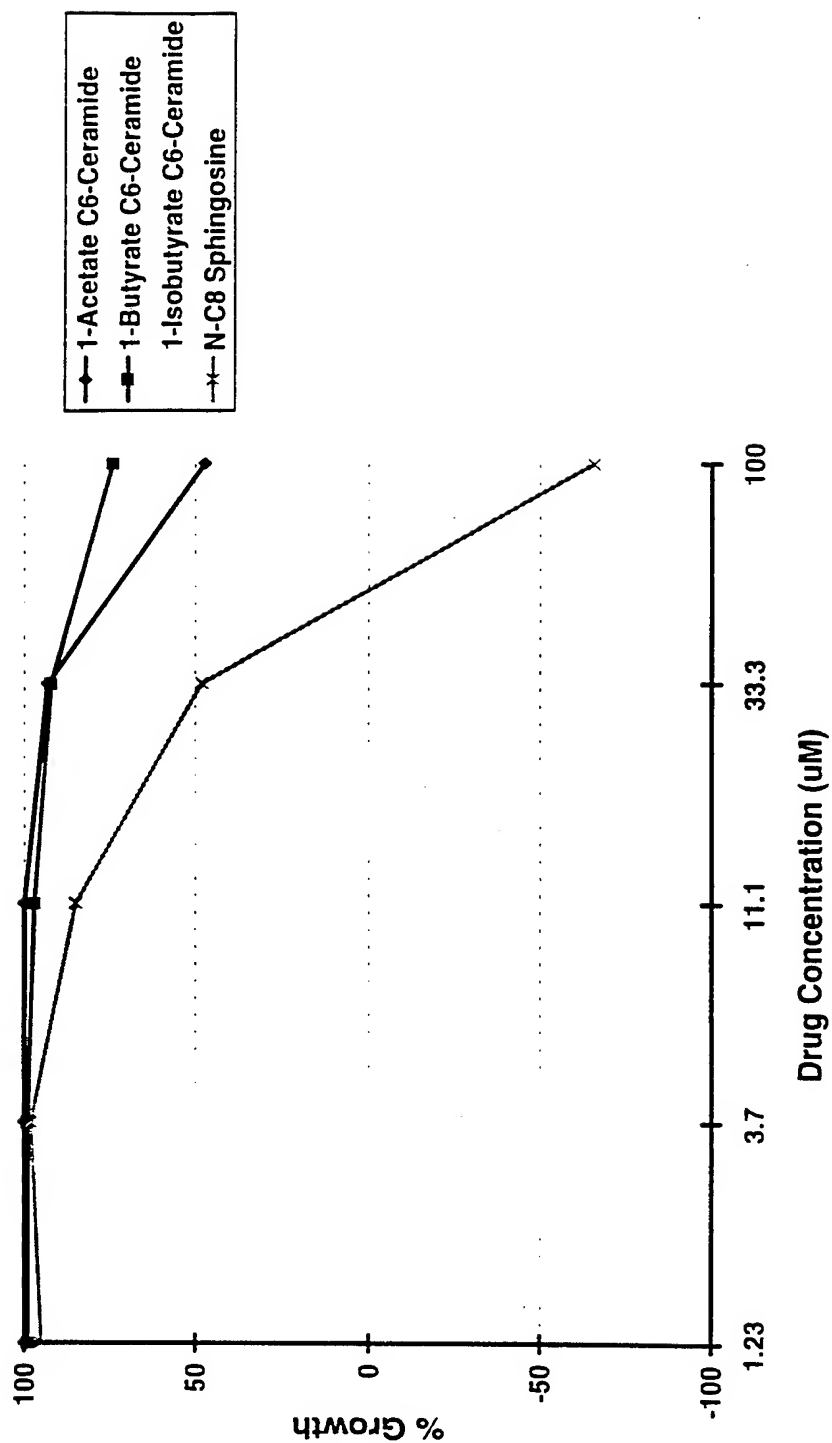
11/21

Fig. 8



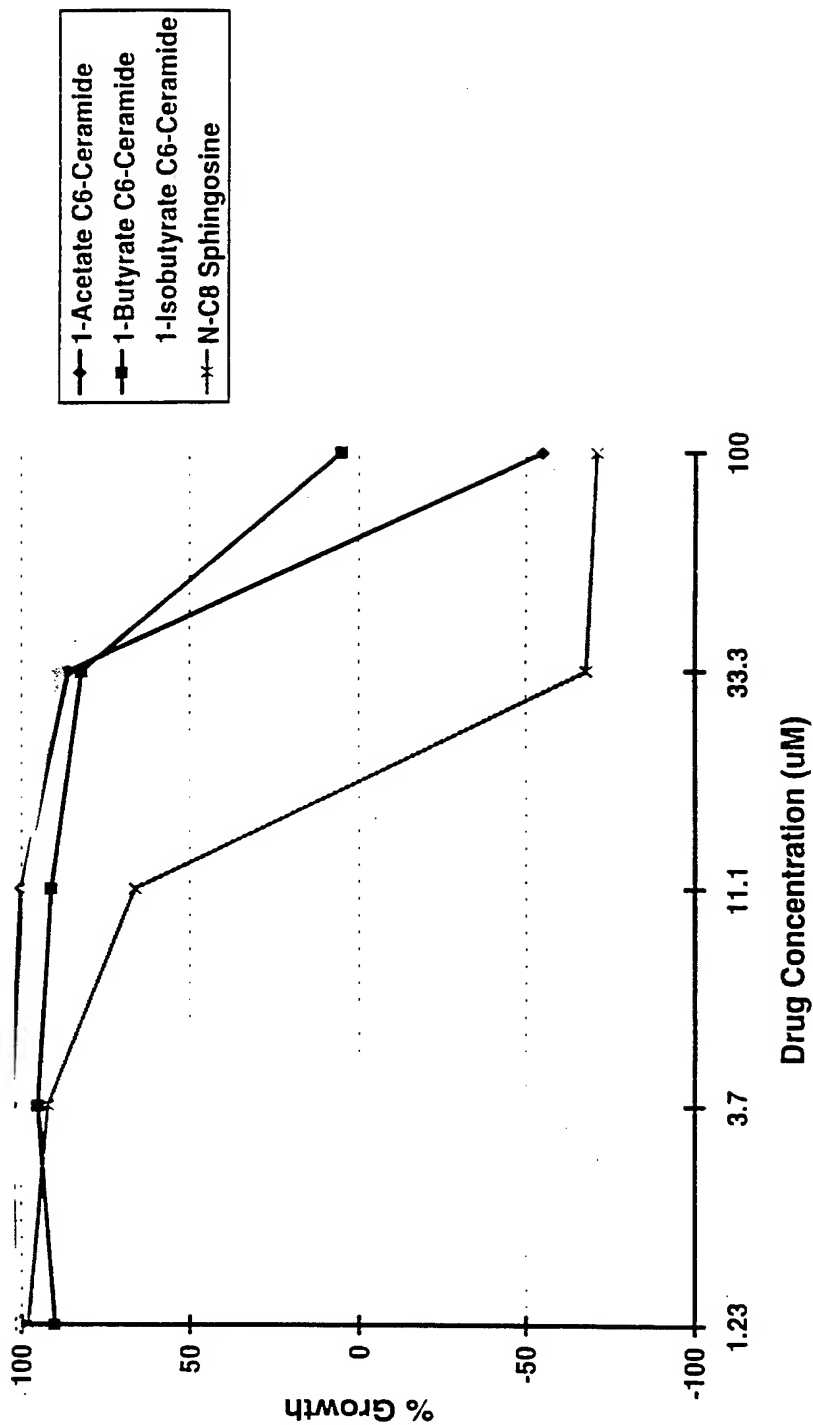
12/21

Fig. 9A



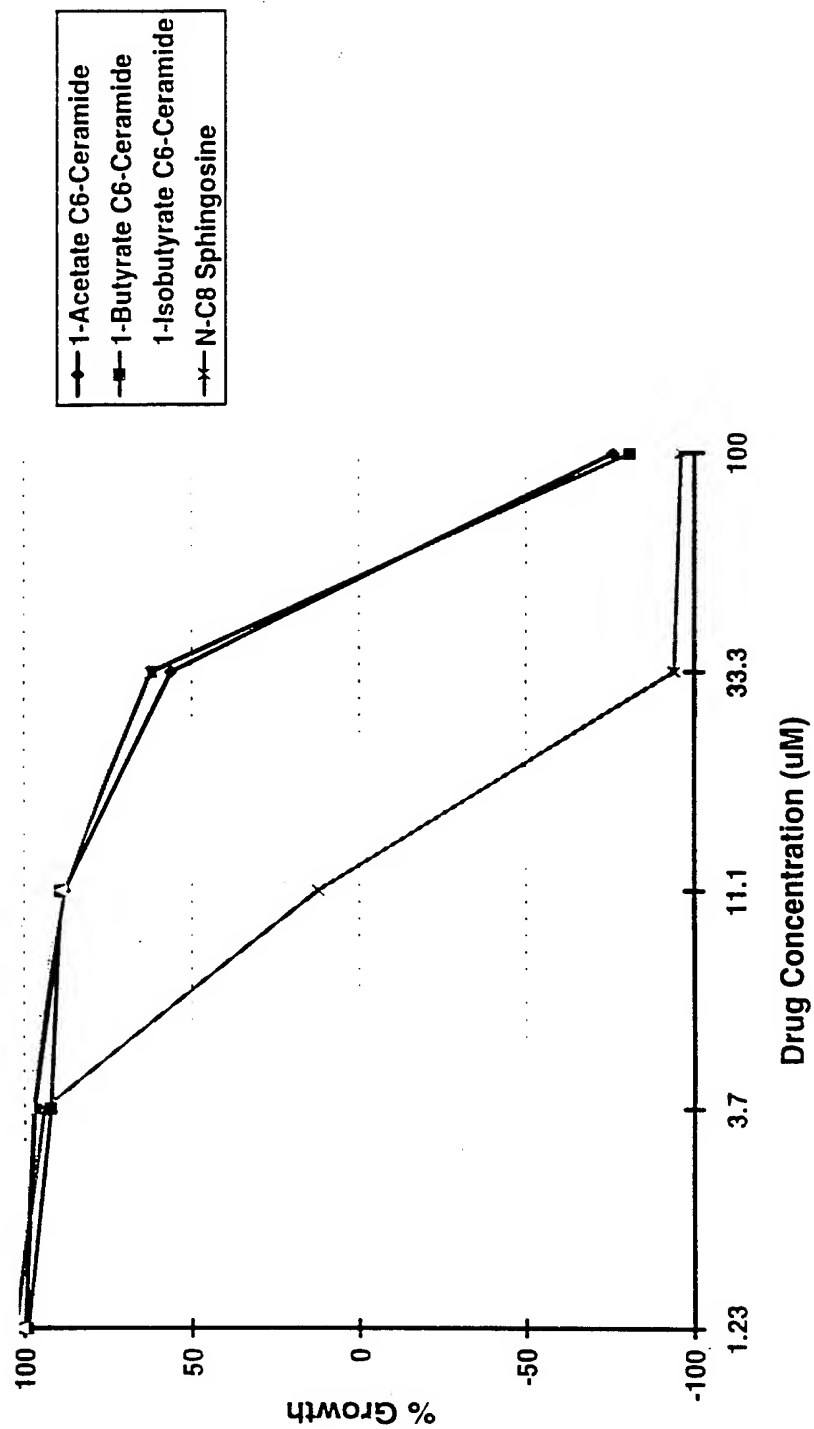
13/21

Fig. 9B



14/21

Fig. 9C



15/21

Fig. 9D

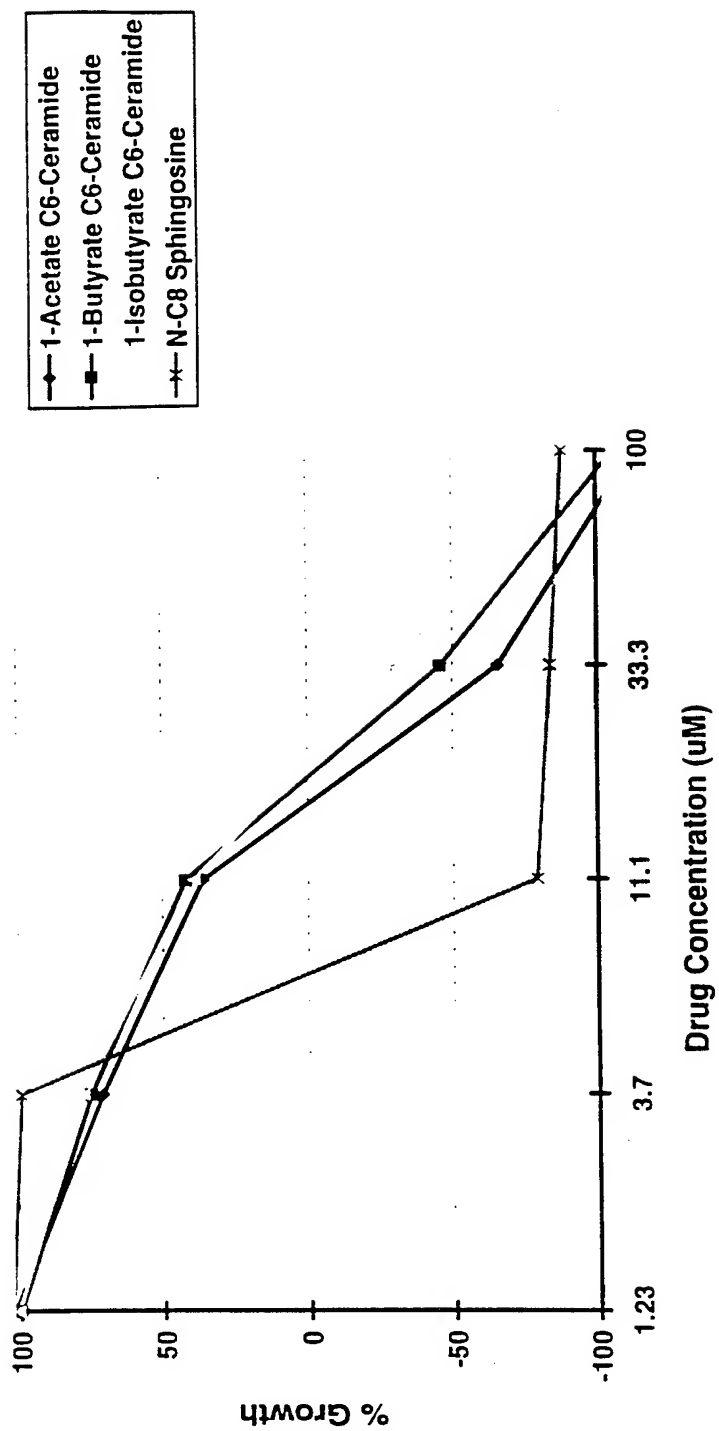
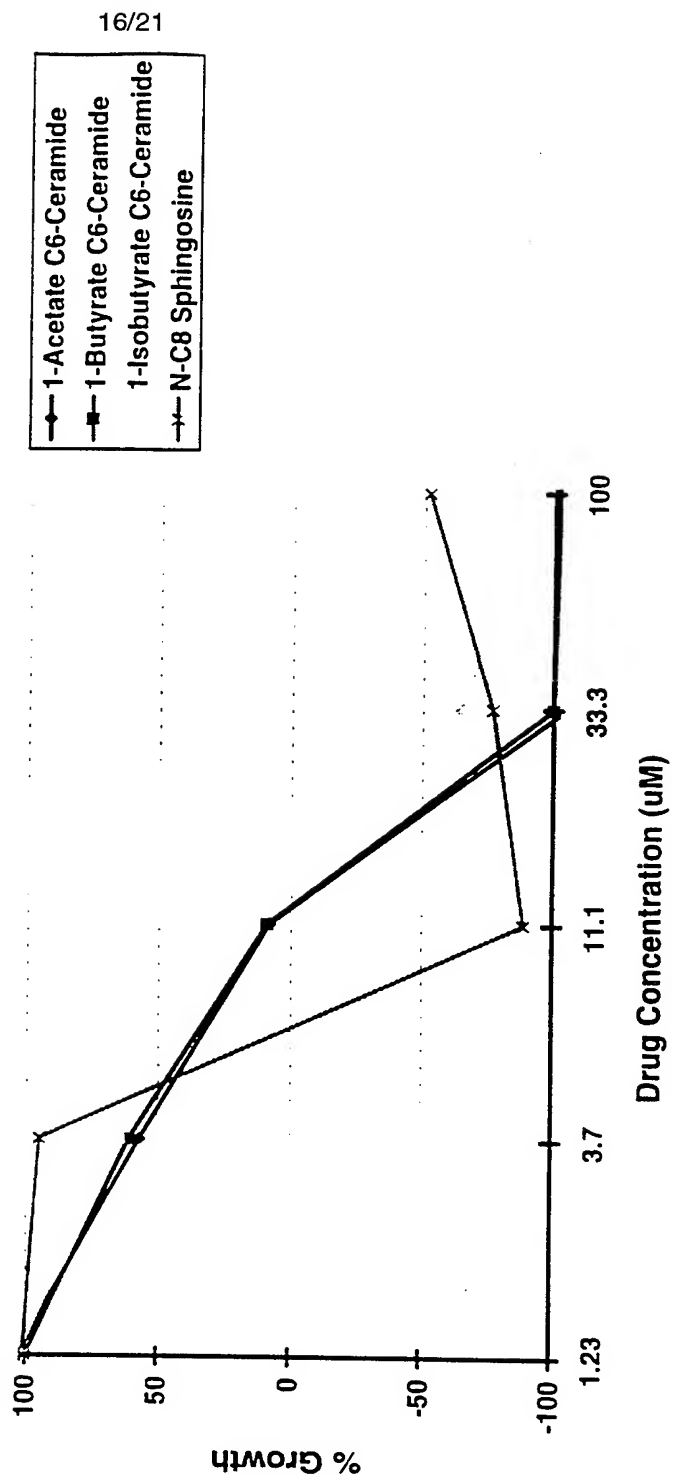




Fig. 9E



17/21

Fig. 10

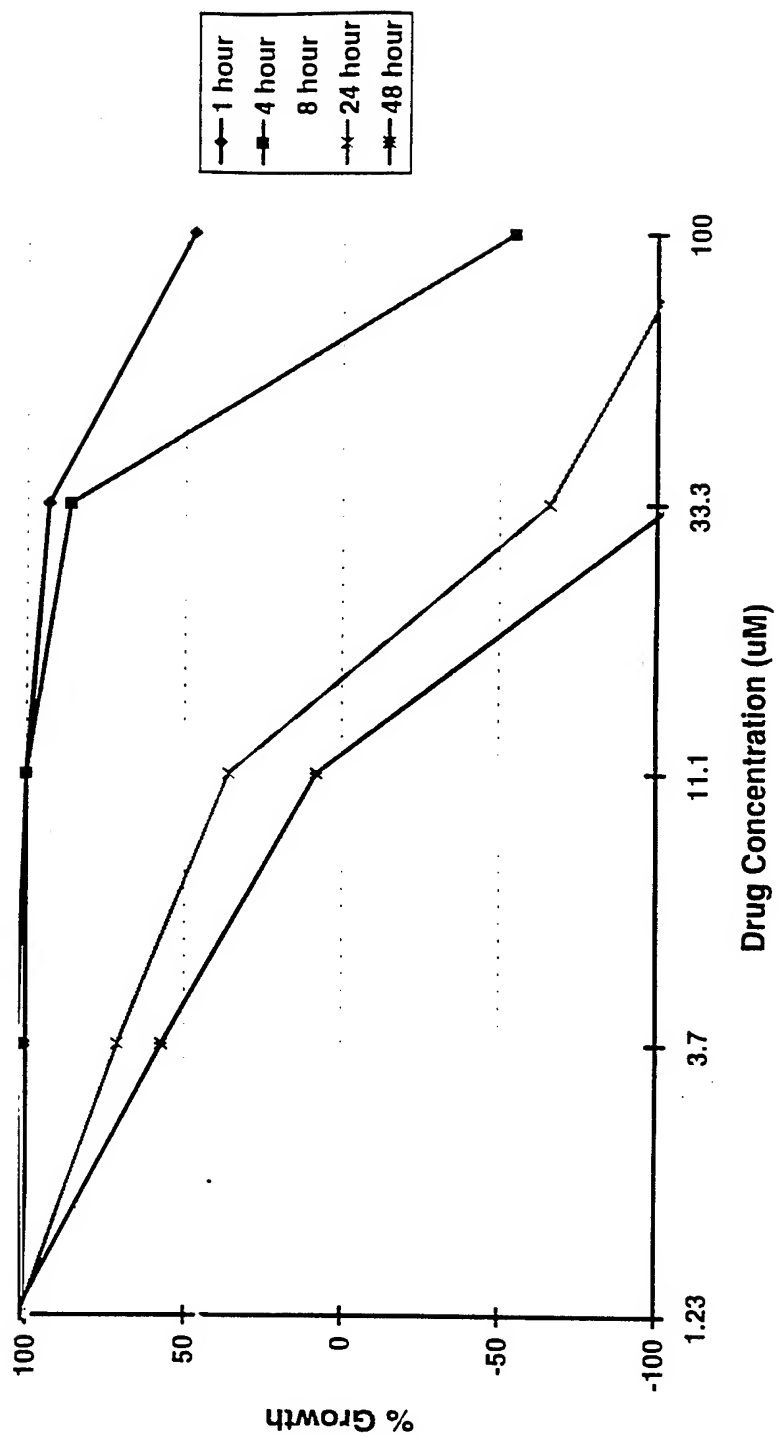
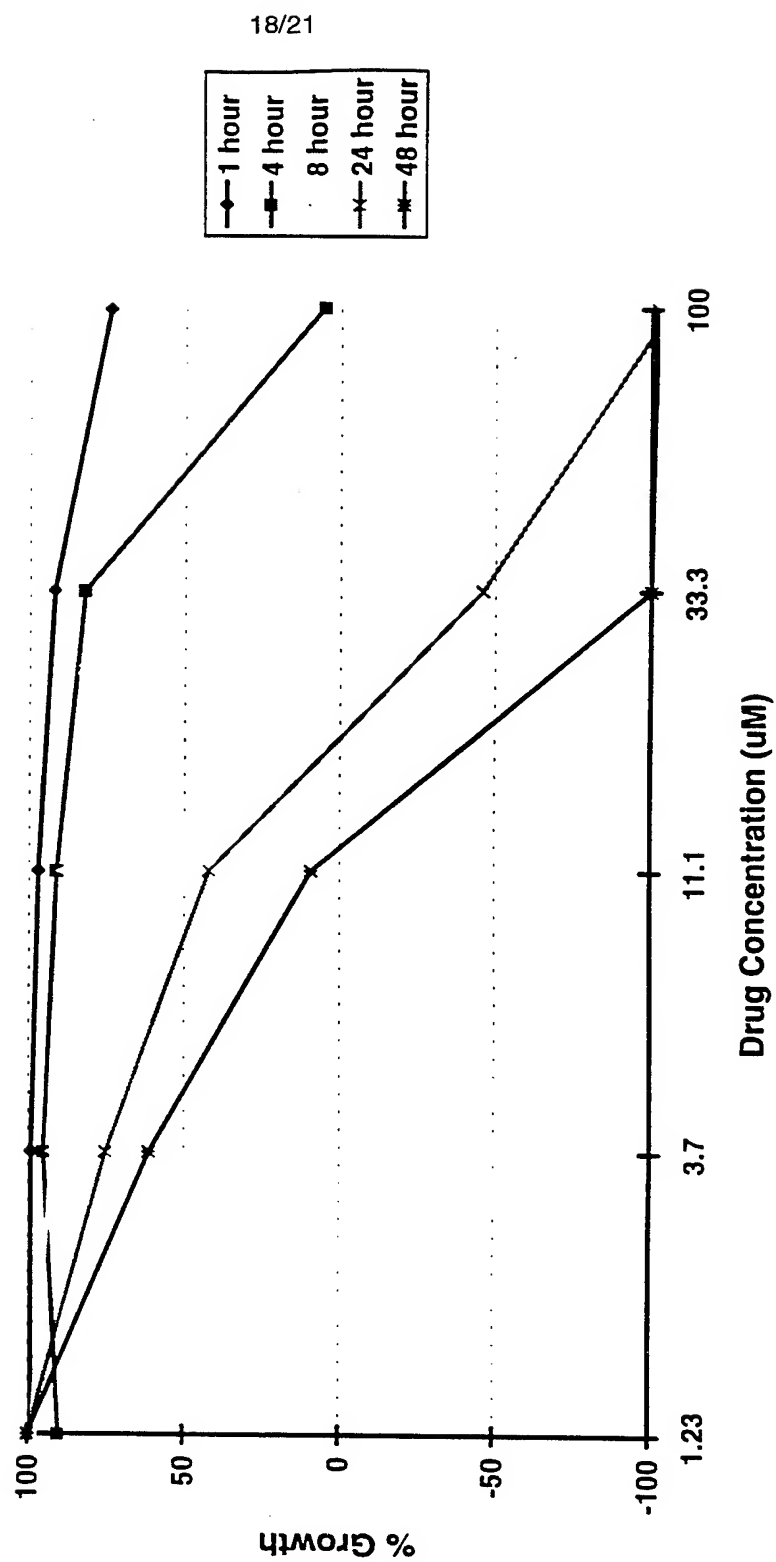
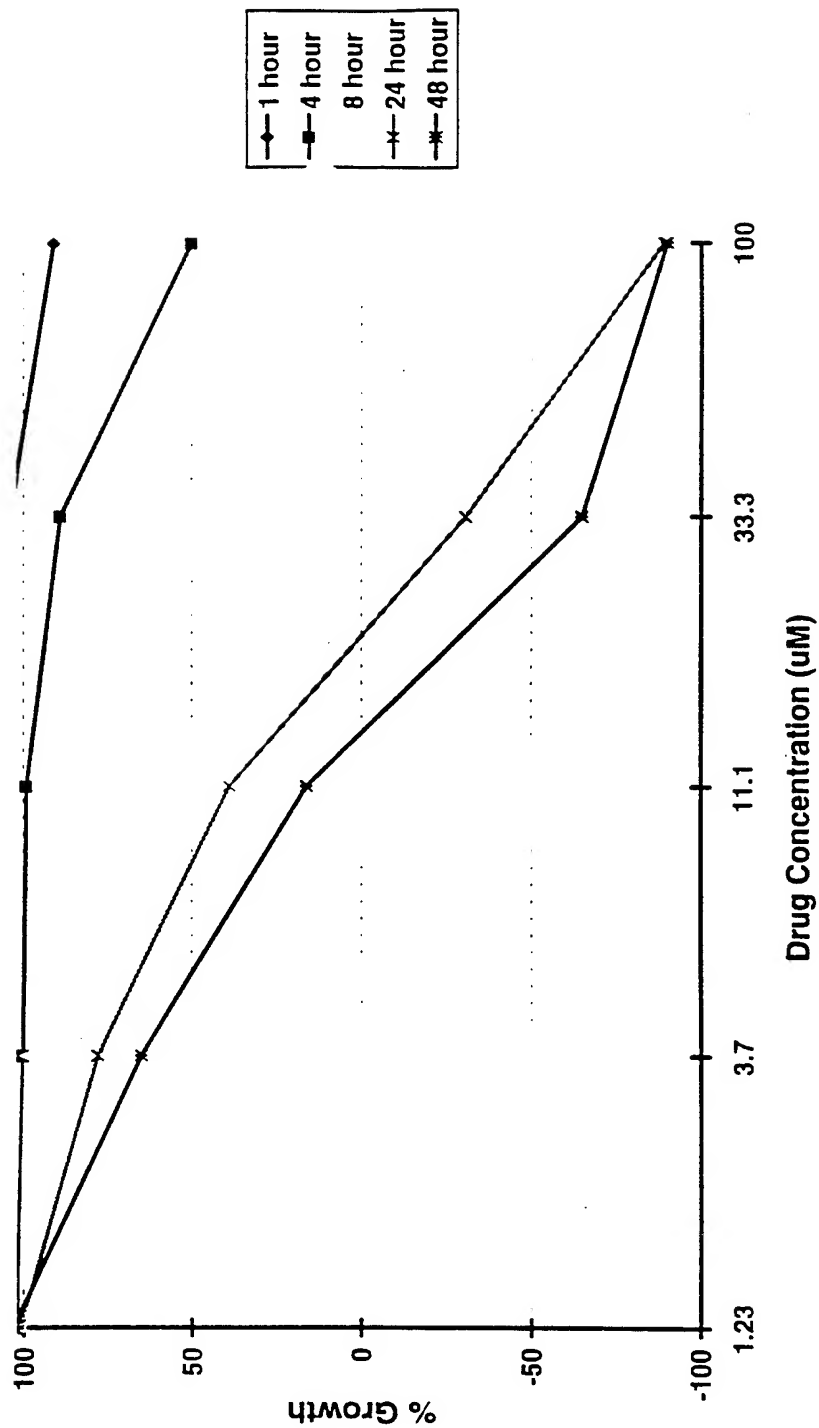


Fig. 11



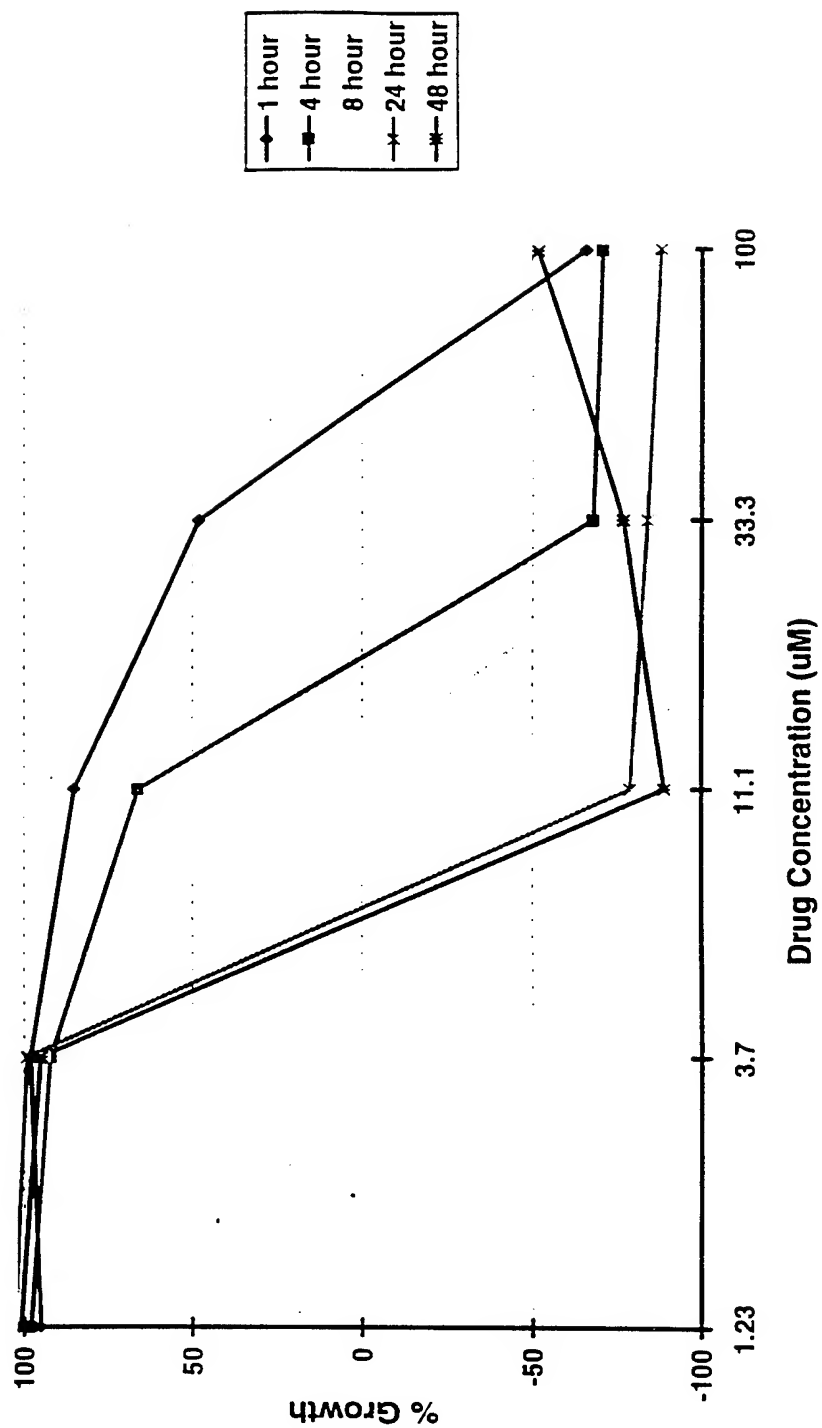
19/21

Fig. 12



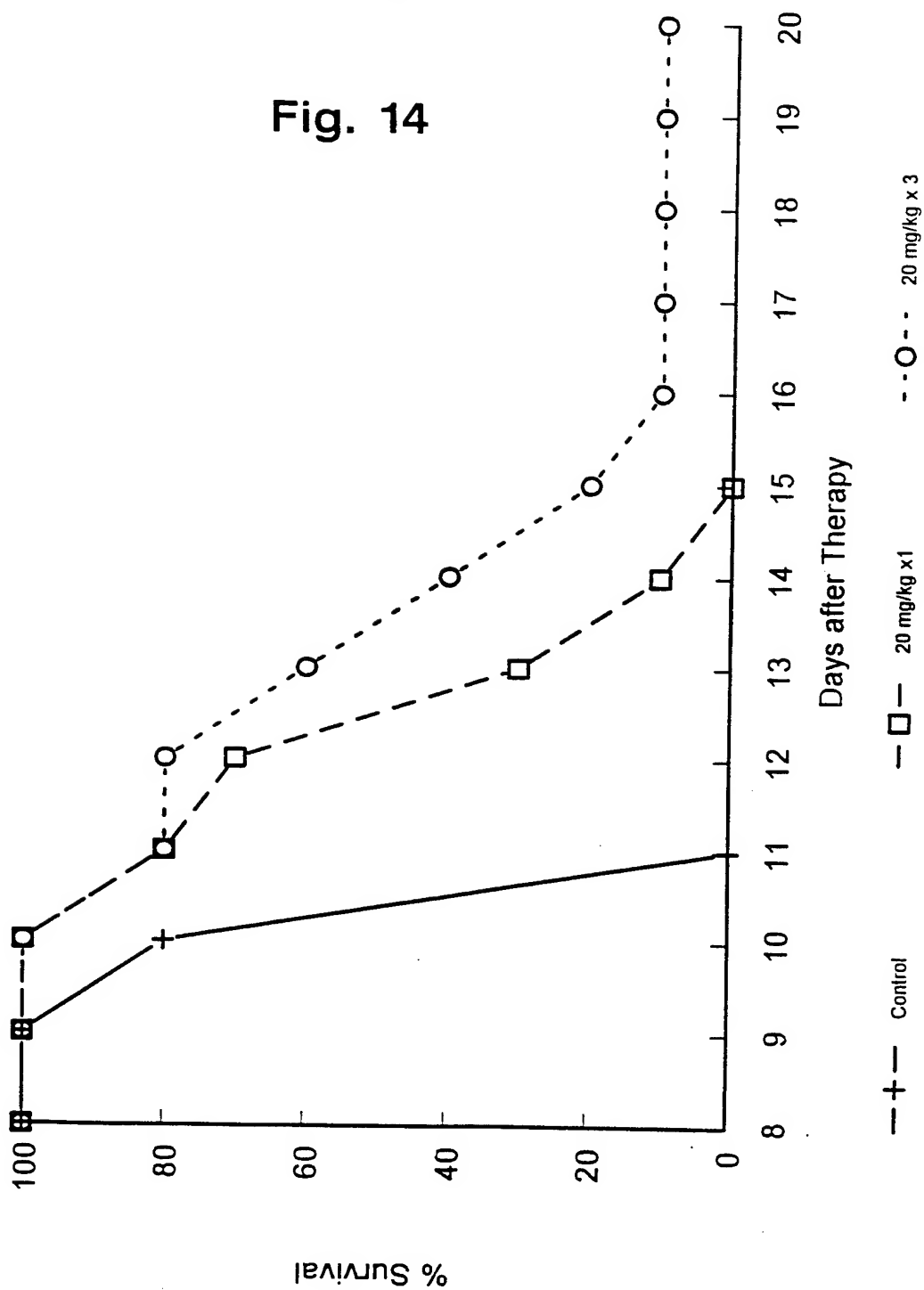
20/21

Fig. 13



21/21

Fig. 14



## INTERNATIONAL SEARCH REPORT

In International Application No  
PCT/US 95/01490

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07F/18 A61K9/127 A61K31/695

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07F A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 112, no. 7, 12 February 1990, Columbus, Ohio, US; abstract no. 56573n, SUGIMOTO, H. ET AL. 'PREPARATION OF SPHINGOSINE DERIVATIVES AS ANTITUMOR AGENTS' page 840 ; see abstract & JP,A,01 093 562 (SHIONOGI AND CO., LTD.) 12 April 1989	1-16
X	TETRAHEDRON LETTERS, vol.29, no.10, 1988 pages 1189 - 1192 OHASHI, K. ET AL. 'SYNTHESIS OF PHOSPHONOSPHINGOLYLIPID FOUND IN MARINE SNAIL TURBO CORNUTUS' * see page 1190: compound (4), (5)*	1-15

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance  
 "E" earlier document but published on or after the international filing date  
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
 "O" document referring to an oral disclosure, use, exhibition or other means  
 "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone  
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.  
 "&" document member of the same patent family

Date of the actual completion of the international search

19 April 1995

Date of mailing of the international search report

24.05.95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+ 31-70) 340-3016

Authorized officer

Rinkel, L

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 95/01490

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEMISTRY, vol.30, 1991 pages 10746 - 10754 GRONBERG, L. ET AL. 'INTERACTION OF CHOLESTEROL WITH SYNTHETIC SPHINGOMYELIN DERIVATIVES IN MIXED MONOLAYERS'	1-15
A	see the whole document ---	27
X	BIOCHEMISTRY, vol.30, 1991 pages 7759 - 7766 KAN, C.-C. ET AL. 'INTERACTION OF CHOLESTEROL WITH SPHINGOMYELIN IN BILAYER MEMBRANES: EVIDENCE THAT THE HYDROXY GROUP OF SPHINGOMYELIN DOES NOT MODULATE THE RATE OF CHOLESTEROL EXCHANGE BETWEEN VESICLES'	1-15
A	see the whole document -----	27



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 95/01490

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP-A-01093562	12-04-89	NONE	